STUDIES ON PROTOZOAL CONTRIBUTION TO THE RUMINANT DIGESTION(Dissertation_全文)

AUTHOR(S):

Ushida, Kazunari

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CONTENTS

ABBREVIATIONS

DM	Dry matter
OM	Organic matter
ADF	Acid detergent fiber
g Ni/ kg OMDR	g nitrogen incorporated into microbial protein par kg organic matter apparently digested in the rumen
VFA	Volatile fatty acid
DAP	2-6 diaminopimelic acid
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
PB	Nucleic acid purine base

INTRODUCTION

Digestion of the ruminant animal is characterized by their specific digestive organ, Rumen, where develops a dense microflora which ferments the diet ingested by the host animal.

It is the rumen fermentation which supplies essential nutrients, energy and nitrogen, and other oligo nutrients such as vitamins, to the host animal.

Foregut digestive organs in the ruminant is generally separated in four sections; rumen, reticulum, omasum and abomasum. Rumen has the most vast capacity of these sections, its volume reaches to 100 - 300 litre in cattle and 4-10 litre in goat and sheep. Thus rumen occupies approximately 70 % of total volume of digestive tract.

Environmental condition in the rumen is relatively constant and defined as follows;

1) temperature ranges from 39°C to 40°C,
2) pH is kept between 5.0 and 7.5,
3) oxidation-reduction potential (Eh) is about -200 mv,
4) nearly totally anaerobic,
5) rich in water (85 to 90 %),
6) regular input of nutrients and saliva, latter has a potent buffering capacity,
7) continual elimination of the end products,
8) continual mixing of contents.

These conditions are optimum for the groups of microorganisms which exist constantly in the rumen. Rumen, thus,can be

considered as a large continuous fermentor.

Ciliate protozoa, anaerobic bacteria, aerobic bacteria, flagellates, fungi, and bacteriophages consist rumen microflora, but former two organisms are dominant; their concentrations are, respectively, 10^5-10^6 per ml and 10^{10} per ml.

It was in mid-19 th century that viable ciliate protozoa in the rumen was observed. Two french workers, GRUBY and DELAFOND (1843) found viable protozoa in the rumen and they also found that these microorganisms take place in the rumen digestion. After their observation, many taxonomical, morphological, physiological studies have been done. The outline of classification of rumen ciliates is given by OGIMOTO and IMAI(1981);

Class, Kinetofragmophora DE PUYTORAC et al.,1974.
Subclass, Gymnostomata BUTSCHLI,1889
Order, Prostomatida SCHWIAKOFF,1896
Suborder, Archistomatina DE PUYTORAC et al.,1974.
Family, Buetschlidae POCHE,1913.
Genus, Buetschlia SCHUBERG,1888.
Subclass, Vestibulifera DE PUYTORAC et al.,1974
Order, Trichostomatida BUTSCHLI,1889.
Suborder, Trichostomatina BUTSCLI,1889
Family, Isotrichidae BUTSCHLI,1889.
Genus, Isotricha STEIN,1859.
Genus, Dasytricha SHUBERG,1888.
Genus, Oligotricha IMAI,1981.

Order, Entodiniomorphida REICHENOW in DOFLEIN et REICHENOW, 1929.

Family, Ophryoscolecidae STEIN,1859.

Subfamily, Entodiniinae LUBINSKY,1957.

Genus, Entodinium STEIN,1859.

Genus, Campylodinium JANKOWSKI,1975.

Subfamily, Diplodiniinae LUBINSKY,1957.

Genus, Diplodinium SCHUBERG,1888.

Genus, Eodinium KOFOID et MacLENNAN,1932.

Genus, Eremoplastron KOFOID et MacLENNAN,1932.

Genus, Eudiplodinium DOGIEL,1927.

Genus, Diploplastron KOFOID et MacLENNAN,1932.

Genus, Polyplastron DOGIEL,1927.

Genus, Elytroplastron KOFOID et MacLENNAN,1932.

Genus, Metadinium AWERINZEW et MUTAFOWA,1914.

Genus, Ostracodinium DOGIEL,1927.

Genus, Enoploplastron KOFOID et MacLENNAN,1932.

Subfamily, Ophryoscolecinae LUBINSKY,1957.

Genus, Ophryoscolex STEIN,1859.

Genus, Epidinium CRAWLEY,1923.

Genus, Epiplastron KOFOID et MacLENNAN,1933.

Genus, Opisthotrichum BUISSON,1923.

Genus, Caloscolex DOGIEL,1926.

These ciliate protozoa, mainly, compose rumen fauna and the composition and the size of rumen protozoal population varies according to the dietary condition and the physiological state of

able 1 Contribution of protozoa to the rumen microbial biomass

Contribution	Animal	Diet	Authors
21% of total rumen-N	Ewe	lucerne hay	WELLER et al.,(1958)
44-53% of the weight of rumen microorganisms	Wether	alfalfa pellet	HUNGATE et al.,(1971)
45% of the protein rumen microorganisms	Cow	alfalfa hay+ barley	IBRAHIM & INGALLS(1972)
60% "	"	" + DES	"
50% "	"	semipurified	"
73% "	"	" + DES	"
40-53% of total rumen-N	Wether	hay + conc.	MICHALOWSKI et al.,(1979)
20-70% rumen microbial-N	-	-	COLEMAN (1979)
33% of total rumen microbial-N	Wether	Cellulose rich	COLLOMBIER (1981)
70% "	"	Starch rich	"
24-46 g of N(pool size of protozoa,*Isotricha,Dasytricha)*	Wether	Oaten chaff+ lucerne+ molasses	LENG et al.,(1981)
1.3-5.7 g of N (pool size of protozoa, *Entodinium.sp.)*	Wether	"	LENG (1982)
45% of protein of rumen microorganisms	Wether	semipurified	HARRISON et al.,(1979)

host, which influence physico-chemical conditions of rumen environment.

Contribution of protozoa to the rumen microbial biomass has been widely studied and 20 to 70 % of rumen microbial nitrogen is attributable to the protozoa (Table 1).Although protozoa are quantitatively important constituents of rumen microbial biomass , their contribution to the duodenal nonammonia nitrogen is fairly small (HARRISON et al.,1979; LENG et al.,1981; LENG,1982; JOHN and ULYATT,1984). Before the observation of WELLER and PILGRIM (1974), it had been widely believed that rumen fluid flows out from rumen without any selected retention of the constituents including microorganisms. However these two Australian workers clearly demonstrated the retention of the protozoa in the rumen, showing that concentration of protozoa in the effluent of reticulum were only 6 to 29% of those measured in rumen fluid. The mechanisms of retention of protozoa are considered as follows; 1) attachement on the large feed particles (BAUCHOP and CLARKE,1976), 2) sedimentation in the ventral sac of the rumen (VALDEZ et al.,1977), 3) attachement on rumen mucosa (ABE et al.,1981).

Protozoal contribution to the intestinal protein supply , thus, must be limited, even their high quality protein (McNAUGHT et al.,1954; WELLER,1957; PURSER and BUECHLER,1966; BERGEN et al.,1968.)

Influence of protozoa on animal growth and production has

Table 2. Effect of defaunation on animal growth and production

	Animals	Diet	method of defaunation	Authors
F=DF Av.wt. at 6m.	Jersey calves (4DF,4F)	Hay:conc=2:1 TDN=60%,CP=23%	segragation from birth	POUNDEN & HIBBS (1950)
F=DF BW gain Feed consump.	Jersey calves (5DF,5F)	Hay:conc=7:3 TDN=54-60%CP=20%	"	HIBBS & CONRAD (1958)
F=DF Wt.gain Feed consump.	4DF calves 4DF lambs 4F calves 21 F lambs	Colostrum→milk+ concentrate, grass or hay	"	EADIE (1962)
F>DF Wt.gain	15 DF lambs 15 F lambs	Colostrum→milk+ conc→milk+conc+ hay→hay+conc. TDN=45% CP=23%	"	ABOU AKKADA & EL SHAZLY(1964)
F>DF DG	15DF lambs 15 F lambs	Hay:conc.=1:1 TDN=65% CP=24%	"	CHRISTIANSEN, KAWASHIMA & BURROUGHS(1965)
F>DF ADG FE	Buffalo calves (18DF, 20F)	milk→ milk+conc TDN of conc.=70% CP of conc.=20%	"	BORHAMI et al. (1967)
F=DF Wt.gain	Cheviot lambs (8DF,8F)	milk→conc+hay	"	CHALMERS et al. (1968)
F=DF Wt.gain	"	"	"	EADIE & GILL (1971)
F=DF	Holstein calves (11DF,11F)	Colostrum→pelleted deit	"	WILLIAMS & DINNUSON(1973)
F<DF Wt.gain	Hereford cattle (18DF,18F)	straw+molasses+ urea, TDN=60% CP=12%	nonyl-phenol-ethoxylate	BIRD &LENG(1978)
F<DF Wt.gain Wool growth	Corridale lambs (24DF,24F)	Oaten chaff+sugar+ urea (+ fish meal) CP=2.8 - 7.9%	"	BIRD,HILL & LENG (1979)
F<DF Wt.gain Wool growth DM intake	20DF lambs 20 F lambs	Oaten chaff+sugar(1:1) + Fish meal+ urea	sodium-lauryl-diethoxy-sulfate	BIRD & LENG(1984)
F<DF ADG	lambs	Sugar beet pulp+ molasses, NaOH straw+molasses	manoxol	DEMEYER, VAN NEVEL& VAN DE VOORDE (1982)

been studied in recent three decades (Table 2). Most of experiments showed that the growth of young ruminants had no clear response to defaunation. Moreover some reports showed negative effect of defaunation on animal growth(ABOU AKKADA and EL SHAZLY,1964; CHRISTIANSEN et al.,1965; BORHAMI et al.,1967). Therefore it had been believed that protozoa might, rather, have a positive effect on ruminant growth. However recent experiments conducted by Australian workers demonstrated a positive effect of defaunation on the growth and production of animals fed high energy and low protein diet(BIRD and LENG,1978; BIRD et al.1979). These results also suggested that protozoa limit a protein supply to the host.

Protozoa have a negative effect of animal growth under certain dietary condition which is characterized both by low protein (low bypass protein) and by high readily fermentable carbohydrate. These dietary conditions suggest that protozoa limit a protein supply to the host by lowering intestinal protein flow and that protozoa affect considerably rumen carbohydrate digestion.

Protein flowing into the small intestine is separated in three fractions;(A) undegraded feed protein in the rumen, (B) microbial protein (bacterial + protozoal), (C) endogeneous protein. The amount of each fraction decides, both qualitatively and quantitatively, intestinal protein which is absorbed by the host and decides naturally the state of protein nutrition of the host.

Therefore effects of protozoa on the amounts of fraction (A) and (B), which are expressed respectively as feed N degradability (% N intake) and efficiency of microbial protein synthesis (g Ni/ kg OMDR), was investigated using defaunated and faunated sheep in this study.

Feed protein degradation in the rumen is affected by following factors; physico-chemical nature of protein, microbial activity and retention time of feed particle in the rumen.

Microbial protein synthesis in the rumen is affected by nitrogen and energy supply, physico-chemical conditions in the rumen and ecological factors such as interspecies relationship between bacteria and protozoa. Relationships between these factors and protozoa were also investigated in this study using in vitro and in sacco techniques.

Protozoa play an important role on the rumen carbohydrate digestion. They affect the efficiency of dietary energy utilization by host through the rumen fermentation ; extent and the pattern of rumen fermentation are affected by the protozoal population. Protozoal contribution to the rumen carbohydrate fermentation has been extensively studied by several groups of worker(EADIE et al., COLEMAN et al., GUTIERREZ et al., JOUANY et al., etc.). However there is not a complete agreement on some points (JOUANY et al.,1981). Protozoal influence on rumen carbohydrate fermentation was, therefore, investigated to elucidate protozoal effect on energetic efficiency of the feed in this

study.

This study was conducted, thus,to study the role of protozoa on rumen digestion and to try to ascertain the advantages and disadvantages of the presence of protozoa for the animal performance.

CHAPTER 1. ROLE OF PROTOZOA ON RUMEN FERMENTATION

INTRODUCTION

Role of protozoa on the rumen fermentation has been widely studied in last 20 years(Table 1-1) and the important contribution of protozoa in butyrate and ammonia production is demonstrated. As to protozoal contribution to other volatile fatty acids, such as acetate and propionate production, there have been some disagreements between results published so far(Table 1-1). Magnitude of effects of defaunation may depend on characteristics of diet given to the experimental animals, since the diet influences not only the size of protozoal population but also the type of bacterial population.

Protozoa produce hydrogen(HUNGATE,1966) which will be further utilized in methane formation. It is, therefore, probable that defaunation promotes a decline of methane production. However the effect of defaunation on the methanogenesis may be detectable under some particular dietary conditions in which effect of defaunation on acetate and propionate is clear,because methane formation is linked with acetate production(WOLIN,1974) and decrease in methane formation coincides with increase in propionate production (DEMEYER and VAN NEVEL,1975). It is likely that protozoal population size is one of the major factors which decide their quantitative contribution to the methane production.

In the present experiment, the effect of defaunation on rumen VFA and gas production was studied using sheep fed two types of diets which promote two different size of protozoal

Table 1-1. Effect of defaunation on ruminal VFA composition

C_2	C_3	C_4	TVFA	Animal	Diet	Authors
↗	↘	-	↘	lambs	R:C=2:1	ABOU AKKADA & EL SHAZLY(1964)
↗	↘	↘	↘	sheep	R:C=1:1	CHRISTIANSEN et al.,(1965)
↗	-	↘	↘	lambs	R:C=4:1	LUTHER et al.,(1966)
-	↘	↗	-	"	R:C=1:4	"
↘	↗	↘	↗	sheep	R:C=2:3	MALES & PURSER,(1970)
↘	↗	↘	↗	heifers	All Conc.	EADIE & MANN (1970)
-	↘	-	↘	lambs	R:C=2:1	EADIE & GILL (1971)
↗	-	↘	-	calves	R:C=2:1	WILLIAMS &DINUSSON (1973)
↗	-	↘	↘	sheep	All Rough.	JOUANY (1978)
-	↗	↘	↘	"	R:C=1:1	"
-	↗	↘	↘	"	R:C=1:1	JOUANY et al.,(1981)
-	↗	↘	↘	"	R:C=3:1	COLLOMBIER,(1981)

R: roughage, C: concentrate

C_2: acetate, C_3: propionate, C_4: butyrate, TVFA: total VFA concentration.

population.

MATERIALS AND METHODS

Animals and Diets

Two Suffolk ewes equipped with a rumen cannula and weighing 60 kg in body weight were used. In experiment 1, 1200 g of alfalfa hay in two equal portions was given to the sheep at 9:00 and 17:00. In the experiment 2, the diet composed of 560 g of alfalfa hay cube, 600 g of rolled barley and 40 g of chopped rice straw was given to the sheep in two equal portions at 9:00 and 17:00. The water and mineral block were always available to the animals.

Defaunation and Faunation

Defaunation was achieved chemically by the method of DEMEYER and VAN NEVEL(1979) using Dioctyl sodium sulfosuccinate(DSS) (Tokyo Kasei,Tokyo); In the previous day of DSS treatment, sheep was fasted. 15 g of DSS dissolved in 300 ml of tap water was introduced into the rumen via a cannula in the morning and then only hay was given to the animal. In the following day, same treatment was done and the complete diet was then given to the animals. In the experiment 2, one ewe died on the first day of DSS treatment. 50 ml of rumen contents from the sheep which had a conventional fauna was introduced into the defaunated rumen via a rumen cannula at the end of defaunation period. All sheep were isolated from other ruminants and kept

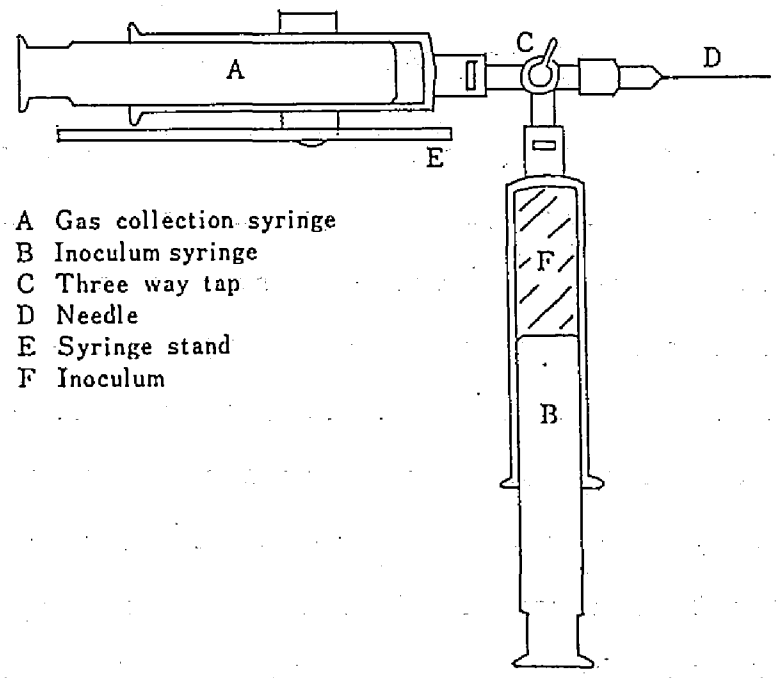
individually in a metabolic cage.

Every two days after the inoculation, protozoal number was counted, and the samples of rumen contents for *in vitro* incubation in faunated period were collected after the protozoal number reaching a plateau value. In both experiments, protozoal number reached a plateau in 14 days after the inoculation.

In vitro incubation

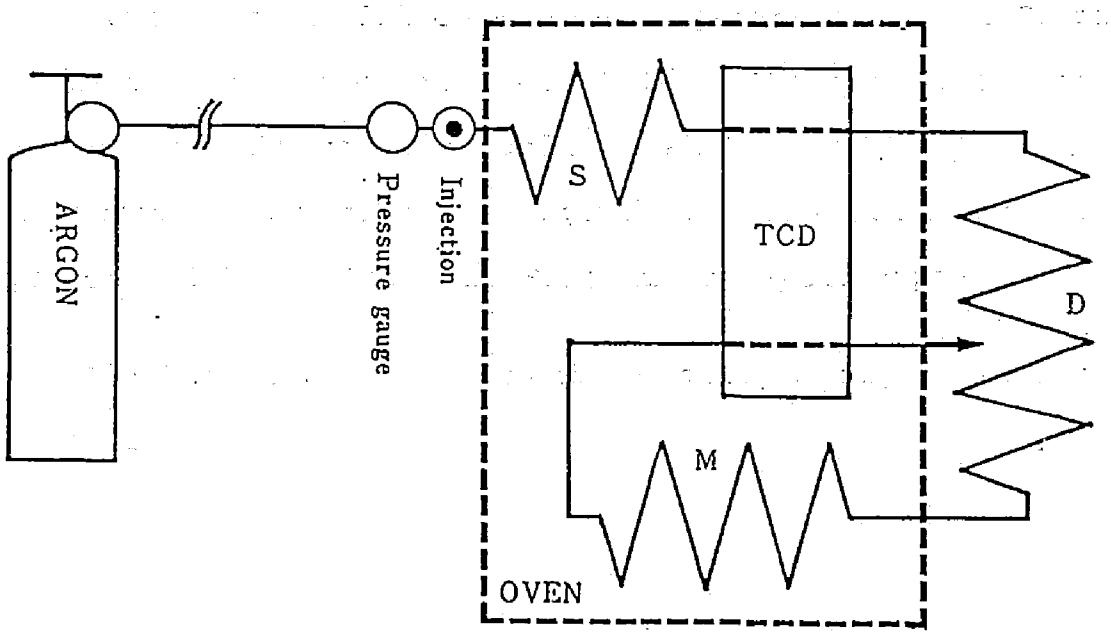
In experiment 1 and 2, approximately 300 ml of rumen contents were taken via a rumen cannula at 6 hours and 4 hours after the morning feeding, respectively. Samples were then filtered through four layers of gauze and 20 ml of filtrate was introduced into a 50 ml syringe which was connected with another syringe by a plastic three way tap (Figure 1-1). The filtrate was then incubated at 39°C for 4 hours. At the end of incubation, 2 ml of 4 N H_2SO_4 was introduced into the syringe to purge the dissolved gas and to stop fermentation. After being well mixed, the gas was totally collected in another syringe and measured for the volume. Gas production at 0 time was measured in the same manner. The amount of gas produced during incubation was obtained by difference between gas volume of the time 4 and the time 0. VFA production was also determined by the same method. In both experiments, 2 times of sampling were made in 2 consecutive days in each period and triplicate incubations were made for each animal.

Figure 1-1. Apparatus for incubation



A Gas collection syringe
B Inoculum syringe
C Three way tap
D Needle
E Syringe stand
F Inoculum

Figure 1-2. Flow diagram of gaschromatography



S: Short column 20 cm × (4 × 3) mm .*stainless column packed with silica gel 60/80
D: Delay column 500 cm × (4 × 3) mm empty stainless column
M: Molecular column 300 cm × (4 × 3) mm stainless column packed with molecular sieve 5A 30/60
* length × (outer diam × inner diam)

Analysis of gas and VFA

Gas composition was analyzed by a gaschromatography (GC-3BT, Shimadzu Seisakusho, kyoto, Japan) with a thermal conductivity detector using a silica gel and a molecularsieve 5A column (Figure 1-2). The conditions of determination were as follows; oven temperature was 50°C, Argon was used as a carrier gas and flowed into a column at the rate of 48 ml/min, 0.5 ml of sample was injected. VFA analysis was done by a gaschromatography (G-180, Yanagimoto Seisakusho, kyoto,Japan) with a flame ionization detector.This system was equipped with two 2 m glass columns (inside diameter was 3 mm) packed with Shimalite TPA(60/80 mesh) coated with 10% PEG 6000. The conditions for the determination were as follows; oven temperature was 150°C, temperature of the injection port was 180°C, Nitrogen was used as a carrier gas and flowed into columns at 1kg/cm^2, 3 µl of sample was injected. These gaschromatographies were connected with an integrator (C-R 2A, Shimadzu Seisakusho, Kyoto,Japan).

All the results were statistically analyzed by the Student's t test.

Table 1-2. Protozoal population in the rumen of faunated sheep (x 10^5/ ml of rumen fluid)

	Experiment 1		*Experiment 2*
sheep No.	80	81	80
Entodinium sp.	2.72	2.48	10.85
Polyplastron multivesiculatum	0.11	0.10	0.67
Diploplastron affine	0.13	0.10	0.58
Ophryoscolex caudatus	0.09	0.08	0.36
Dasytricha ruminantium	-	-	0.12
Isotricha sp.	0.11	0.10	0.03
Total	3.16	2.86	12.61

RESULTS

Protozoal population in a sampling period is shown in Table 1-2. In experiment 1, approximately 3.0×10^5 of protozoa/ml of rumen fluid was maintained, while 12.6×10^5 of protozoa/ml of rumen fluid which is considered as a large population was established in experiment 2. Fauna were similar in both experiments, composing Entodinium sp., large Ophryoscolecidae and Isotrichidae with population ratios of 85-90%, 10% and 2-3 %, respectively.

In vitro VFA production rate was affected by the defaunation (Table 1-3) ; decrease in acetate and butyrate and increase in propionate. Pattern of changes were similar in experiment 1 and 2, but the magnitude of effect of defaunation was greater in experiment 2 than in experiment 1. In vitro gas productions (Table 1-4) were not clearly affected by defaunation in experiment 1, while carbon dioxide and methane production rates were significantly decreased in experiment 2. Hydrogen was not detected in experiment 1 and only a trace amount in experiment 2.

DISCUSSION

Effect of defaunation on in vivo rumen VFA molar ratio was focused in many reports. These bibliographical data were difficult to compare each other, because the differences in species or age of the experimental animal used, and in amount or

Table 1-3. Effect of protozoa on ruminal VFA production in vitro

(μM/ ml of rumen fluid/ hour: means±s.d.;n=6)

sheep No.		DF	F
		Experiment 1	
	C_2	1.97±0.10	2.56±0.35*
80	C_3	0.63±0.04	0.56±0.08
	C_4	0.43±0.02	0.66±0.10**
	other acids	0.26±0.22	0.33±0.04*
	Total	3.29±0.17	4.11±0.37**
	C_2	2.11±0.10	2.85±0.11**
81	C_3	0.68±0.04	0.62±0.01*
	C_4	0.47±0.02	0.64±0.01**
	other acids	0.28±0.01	0.35±0.01**
	Total	3.54±0.20	4.46±0.11**
		Experiment 2	
	C_2	2.64±0.43	4.81±0.86**
80	C_3	1.28±0.13	0.79±0.09**
	C_4	0.74±0.29	1.22±0.31*
	other acids	0.39±0.05	0.28±0.05*
	Total	5.05±0.82	6.82±1.30*

DF: defaunated period, F: faunated period.
C_2: acetate, C_3: propionate, C_4 butyrate, other acids: iso butyrate, iso valerate and valerate. * $p<0.05$,** $p<0.01$

Table 1-4. Effect protozoa on ruminal gas production *in vitro* (ml x 10^{-2}/ ml of rumen fluid/ hour, means±s.d.,n=6)

sheep No.		DF	F
		Experiment 1	
	CO_2	9.97±1.00	10.04±0.93
80	H_2	nd	nd
	CH_4	2.11±0.14	1.99±0.42
	CO_2	7.92±2.32	10.19±2.45
81	H_2	nd	nd
	CH_4	2.01±0.10	2.16±0.20
		Experiment 2	
	CO_2	13.26±1.33	17.88±1.23**
80	H_2	trace	trace
	CH_4	5.94±1.51	8.52±0.33**

DF: defaunated period, F: faunated period. **p<0.01

nd: not detected

quality of the experimental diet were not ignorable. Since effect of these experimental conditions on rumen fermentation pattern is very complex , effect of defaunation may not be distinguished from other effects in many case. In spite of the considerable difference in the experimental condition, protozoal contribution to the butyrate is always demonstrated and present results were also in line with these previous results. Protozoa produce acetate and butyrate(HUNGATE,1966). It is, therefore,clear that their contribution to butyrate is quantitatively important in most cases and that bacterial population which produces this acid can not fully replace protozoal butyrate production after the defaunation. Defaunation decreased acetate production significantly in this experiment. This decrease derived from either incomplete compensation by bacteria for disappeared protozoal acetate production or decrease in bacterial activity, especially acetate producer, by defaunation as suggested by KURIHARA et al.(1968). However the extent of decrease in acetate production was small with all forage diet in the experiment 1 and fermentation pattern was not greatly modified by defaunation. All forage diet supported acetogenic fermentation independently of protozoa.

Since protrozoal propionate production is fairy small (HUNGATE,1966), production of this acid is almost wholly attributable to the bacterial population. The increase in propionate molar ratio observed in the experiment 2 might arise from the relative increase in population size or activity of propionate

producing species. Effect of defaunation on the composition of bacterial population was scarcely reported. One of the reports showed the increase in population size of Bacteroides(BRYANT and SMALL,1960) and other report showed the increase in population size of starch digesting species (KURIHARA et al.,1978). Protozoa have a strong preference for the starch. The proliferation of the starch digesting bacteria is suppressed by the presence of a nutritional competition between protozoa and these bacteria and also by the protozoal selective ingestion of these bacteria which attach on the starch granule. Starch digesting bacteria produce lactate, succinate or propionate; former two products are further catabolized to propionate by other bacteria such as Selenomonas ruminantium (HUNGATE,1966). Therefore the decrease in population size of these bacterial group promote the decline of propionate production. Such a change in bacterial population probably occured in the experiment 2. Since the increase in propionate production induced by defaunation was only slight, drastic development of propionate producer might not occur after defaunation in the experiment 1. The nature of all forage diet, which was not expected to support a large propionate producing bacterial flora, probably lead this contrasted results. These results suggest that protozoa play an important role on the establishment of acetogenic fermentation when animal is fed a high concentrate diet and has a large protozoal population.

Present results showed that total VFA production rate was

decreased by defaunation. In vivo total VFA concentration is often decreased by defaunation(Table 1-1,4-2). Defaunation decreases ruminal OM digestion(Table 4-4, LINDSAY and HOGAN,1972) and this decrease provokes a decline in total VFA production. This decrease is also explained, in part, by the disappearance of protozoal acid production. While bacterial population size is generally larger in defaunated rumen than in faunated rumen. According to the observations of previously published reports, there was probably a larger bacterial population in defaunated rumen (EADIE and HOBSON,1962; KURIHARA et al.,1968, 1978; EADIE and GILL,1971; WHITELAW et al.,1972; DEMEYER and VAN NEVEL,1979). Such an increase can compensate the disappearance of protozoal acid production. The extent of increase in bacterial population size is often considerable and one of the reports showed five times larger population size (DEMEYER and VAN NEVEL,1979). In spite of these large increases in bacterial population size, VFA concentration is smaller in defaunated rumen and meanwhile in vitro VFA production in this study was also lower. It is, therefore, probable that protozoal acid production is important and that the activity of bacteria is lowered by the disappearance of protozoa as pointed out previously (KURIHARA et al.,1968). Effect on VFA production rate (Table 1-3) was clearer in experiment 2 than in experiment 1, especially on the propionate production rate. Since the sampling time were not same in these two experiments, it is not easy to compare each other. However amplified

effect of defaunation on propionate production rate in the experiment 2 was due to the large protozoal population and also to the diet used in this experiment which promotes large population of starch digesting bacteria in the absence of protozoa.

Negative effect of defaunation on methane production was reported by JOUANY et al.(1981) who indicated the increase in the CO_2/CH_4 ratio. More recently, WHITELAW et al.(1984) demonstrated the antimethanogenic effect of defaunation in steer maintained in respiratory chambers and ITABASHI et al.(1984) also demonstrated same effect of defaunation on methane production in goat.

In experiment 2, faunated sheep had a size of protozoal population similar to the one reported by JOUANY et al., WHITELAW et al. and ITABASHI et al. which is considered as a large population. In experiment 1, the population size was smaller and the effects of defaunation on methane production rate were less clearer. These results suggested that protozoal contribution to the ruminal methanogenesis is important when their population size is larger than 1×10^6 organisms/ ml of rumen fluid. Protozoa, itself, do not produce methane but they may supply hydrogen to methanogenic bacteria. Moreover methanogenic bacteria attach on the protozoal surface(STUMM et al.,1980); protozoa then supply them the site for living. Defaunation implies, therefore, the disappearance of supplies of hydrogen and site for living to methanogenic bacteria. Hydrogen supply by protozoa may not be important compared with bacterial supply and may be readily

compensated by bacteria, when high roughage diet is given. Meanwhile their hydrogen supply may be important with high concentrate diet, especially given in a restricted amount, which promote a large population ($> 1 \times 10^6$). If total VFA production is not suppressed by defaunation, antimethanogenic effect of defaunation then improves energetic efficiency of diets.

SUMMARY

Role of protozoa on ruminal gas and VFA production was studied by *in vitro* incubation technique using two fistulated ewes. The experiment was consisted of two periods, defaunated and faunated, and sheep received two different diets, all forage (AF) and forage : concentrate = 50 : 50 (FC), which promoted different sizes of protozoal population. FC supported a large population, while AF promoted smaller one.

Defaunation decreased acetate and butyrate production rate, while it increased propionate production rate. Defaunation, however, decreased total VFA production. These effects were observed with both diets, but were clearer with FC diet. It is suggested that protozoa play an important role on the establishment of acetogenic fermentation in the rumen of animal fed a substantial amount of concentrate diet which support a development of large protozoal population. Defaunation decreased methane and carbon dioxide production rate with FC diet, however it had no clear effect on these gas production with AF diet. It is suggested that protozoal hydrogen supply to the methanogenesis is quantitatively important when large population of protozoa is supported by substantial concentrate feeding.

CHAPTER 2. ROLE OF PROTOZOA ON RUMEN PROTEIN DEGRADATION

INTRODUCTION

The role of protozoa in the ruminal digestion of protein is not well known. It has been reported that their presence in the rumen causes an increase in ammonia-nitrogen (JOUANY and SENAUD, 1982, 1983) and that their contribution to the amount of microbial protein entering the small intestine is low (WELLER and PILGRIM, 1974; COLLOMBIER,1981). Recently, other authors have shown that the duodenal flows of amino acids (VEIRA et al.,1984) were significantly increased in sheep with no protozoa present in the rumen.

The purpose of this experiment, using the techniques "in vitro" and "in sacco", was to determine the role of rumen protozoa in the degradability of feed protein. Feed protein degradability is affected by (1) physico-chemical characteristics of feed protein, (2) microbial activity and (3) retention time in the rumen.

Feed nitrogen(crude protein) is fractionized in true protein and in non protein nitrogen (NPN) such as amides, amines, amino acids, nucleic acid, urea, ammonium salts, nitrate and so on. True protein fraction is separated in two components, soluble and insoluble. NPN fraction is normally totally degradable in the rumen. While true protein has different degradability according to its solubility, because soluble protein and insoluble protein have different mode of degradation in which roles of bacteria and of protozoa are different. For the soluble protein degradation,

bacterial role may be more important, while protozoa may play more important role on insoluble protein degradation. This study was, therefore, conducted to elucidate the role of protozoa on rumen feed protein degradation with special reference to the physico-chemical nature of protein.

MATERIALS AND METHODS

Preparation of a defaunated sheep

The defaunation was done as described by JOUANY and SENAUD (1979a). The first morning, all the rumen contents is collected via a rumen cannula in a backet and is frozen at - 15°C. The rumen is then washed with tap water at 39°C until water is perfectly clear. A solution of 10 ml of formaldehyde (30%) in 10 litres of water is introduced into the rumen and all the mucosa is in contact with the solution for a short time. The rumen is then quickly emptied and carefully washed with tap water for 10 minutes. The rumen is finally filled with tap water. The sheep is fasted from this moment (only water is given). Each morning the next two days, the rumen is washed with tap water, treated with formaldehyde solution and rinsed in the same way as that used on the first day. During the fasting period (3 days), 500 ml of an acetic acid solution (5%), a vitamin complex (Vitaperos, a half of a sack per day, Iffa Merieux, Lyon, France) and liver protector (Hepatransyl, 1 spoonfull per day, Lathevet, Paris, France) are introduced into the rumen twice a day and vitamin B complex-

es (Complex B fort, 2 ml, Proligo, Paris, France; Terneurine, 1 flacon, Allard, Paris, France; Vitamin B12, 1 ml, Vetoquinol, Lure, France) is also injected intramusculary twice a day. In the afternoon of the third day, the thawed rumen contents which is collected on the first day are reintroduced into the rumen and food is given to the sheep. Although the sheep eat, in general, spontaneously and rumen motility appears quickly, sometimes roughage must be introduced into the rumen via a cannula and the injection of Prostimidine (Roche, Neuilly sur Seine, France) must be made in order to recover the gastro-intestinal motility. The sheep is then normally fed in avoiding the contacts with other ruminants.

Animals and Diets

Five defaunated and six faunated Texel wether sheep (average wt. 60 Kg) were used as rumen contents donors during the in vitro study. Three defaunated and three faunated sheep were used during the in sacco measurements. Each sheep was fitted with a rumen cannula (ϕ 60 mm) and was individually penned.

Each sheep received a daily ration containing 800g of pelleted lucerne hay, 200g of chopped wheat straw, 250g of mixed grass hay, and 150g of pelleted barley. The diet was given in two equal portions at 12 hour intervals. Water and a mineral block were always available to the sheep except during the night before rumen content sampling for the in vitro studies.

In vitro determination of protein degradability

In vitro degradabilities of lupine seed, peanut cake, soybean cake, and fish meal proteins were measured with either defaunated or faunated rumen contents using the method of VERITÉ and DEMARQUILLY (1978) as modified by JOUANY and THIVEND (1985). This in vitro incubation system(Figure 2-1) consists on a water bath in which 8 erlenmyer flaskes of 1 litre are immersed. The flask contains an inoculum and source of energy and nitrogen. A polyethylene tube, 3 mm interior diameter, fixed externally to a rigid polyvinyl chloride tube the end of which is ponctured with several holes and covered by three layers of gauze. A syringe located at the end of polyethylene tube allows liquid to be sampled during fermentation. Except for the sampling time, this tube is closed by a clamp. The flask is connected by a polyethylene tube to an inverted graduated two litre test cylinder which is filled with the 30 % $CaCl_2$ solution in the water bath. A T-shaped plastic three way tap is equipped into the tube for the gas sampling. Approximately 500 ml of rumen contents were taken from each animal before morning feeding and well mixed in a flask which is previously heated at 39°C. Half of contents are filtered through a metal sieve with 25 mesh(1 mm^2). Then 100 ml of filtrate, 100 ml whole contents ,200 ml of artificial saliva(1) saturated in CO_2 at 39°C and 5 ml of 17.6% of

(1) artificial saliva ingredients for 1 litre of distilled water

$NaHCO_3$	9.24g
$Na_2HPO_4 \cdot 12H_2O$	7.13g
NaCl	0.47g
$MgCl_2 \cdot 6H_2O$	0.10g
$CaCl_2 \cdot 2H_2O$	0.07g
KCl	0.45g

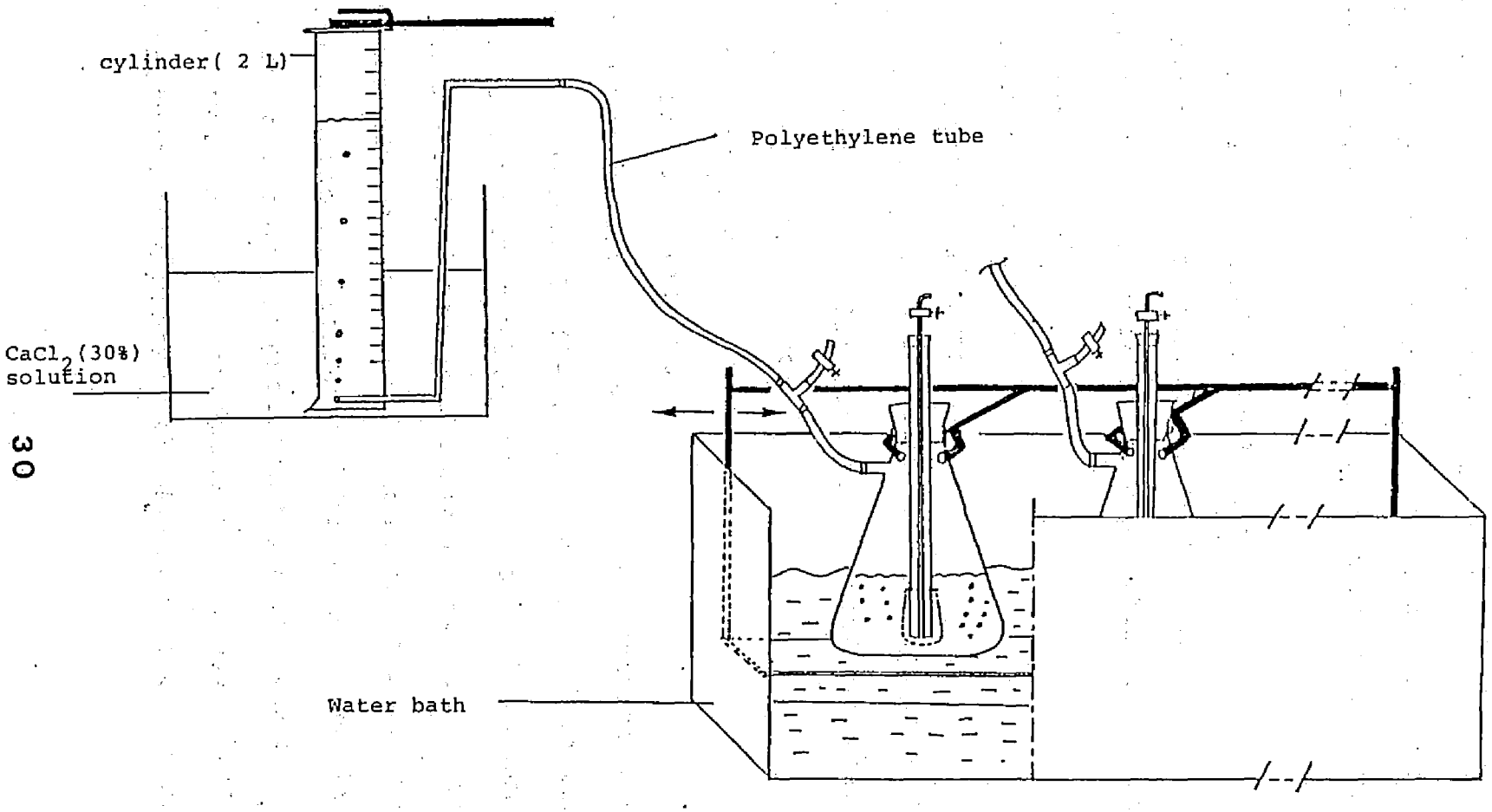


Figure 2-1 In vitro incubation system

ammonium sulfate solution were introduced into a fermentor flask. x grammes of nitrogenous feed containing 125 mg of nitrogen and y grammes of corn starch so that x + y represents 13 g of dry matter were also introduced into a flask as a energy and nitrogen source.

8 fermentor flaskes were allotted as follows ;

1) control incubation(A): corn starch + inoculum(taken from defaunated animal)+ ammonium sulfate solution + artificial saliva,

2) control(A) + nitrogen source no.1,

3) control(A) + nitrogen source no.2,

4) control(A) + nitrogen source no.3,

5) control incubation(B): corn starch + inoculum(taken from faunated animal)+ ammonium sulfate solution+ artificial saliva,

6) control(B) + nitrogen source no.1,

7) control(B) + nitrogen source no.2,

8) control(B) + nitrogen source no.3.

Calculation of protein degradability was conducted using the formula of $F=V/N((A-B)+M/G(D-E))$ (V: volume of incubated fluid, N: amount of nitrogen supplied by tested feed, A: ammonia concentration in a sample flask at the end of incubation(6h),B: ammonia concentration in a control flask at the end of incubation, M: amount of ammonia disappeared between 1h and 6h in a control flask, G: volume of gas produced between 1h and 6h in a control flask, D:volume of gas produced in a sample flask, E: volume of gas produced in a control flask.) Each protein source

was incubated in both defaunated and faunated rumen contents on the same day; at least five determinations were made for each protein source.

Determination of azocasein and casein degradation rate

Azocasein degradation rate with defaunated and faunated rumen contents was measured as described by BROCK et al.(1982), withtwelve determinations for each group of sheep. Casein degradation rate with defaunated and faunated rumen contents was measured by the method of ERFLE et al.(1982).

In Sacco Study

The nylon bag method was used for the measurement of soybean cake protein degradability in defaunated and faunated sheep rumens. Heat sealed bags measuring 11x6.5 cm were made of nylon cloth having a calibrated pore size of either 50 or 100 µm (Blutex xx, TRIPETTE & RENAUD, PARIS).

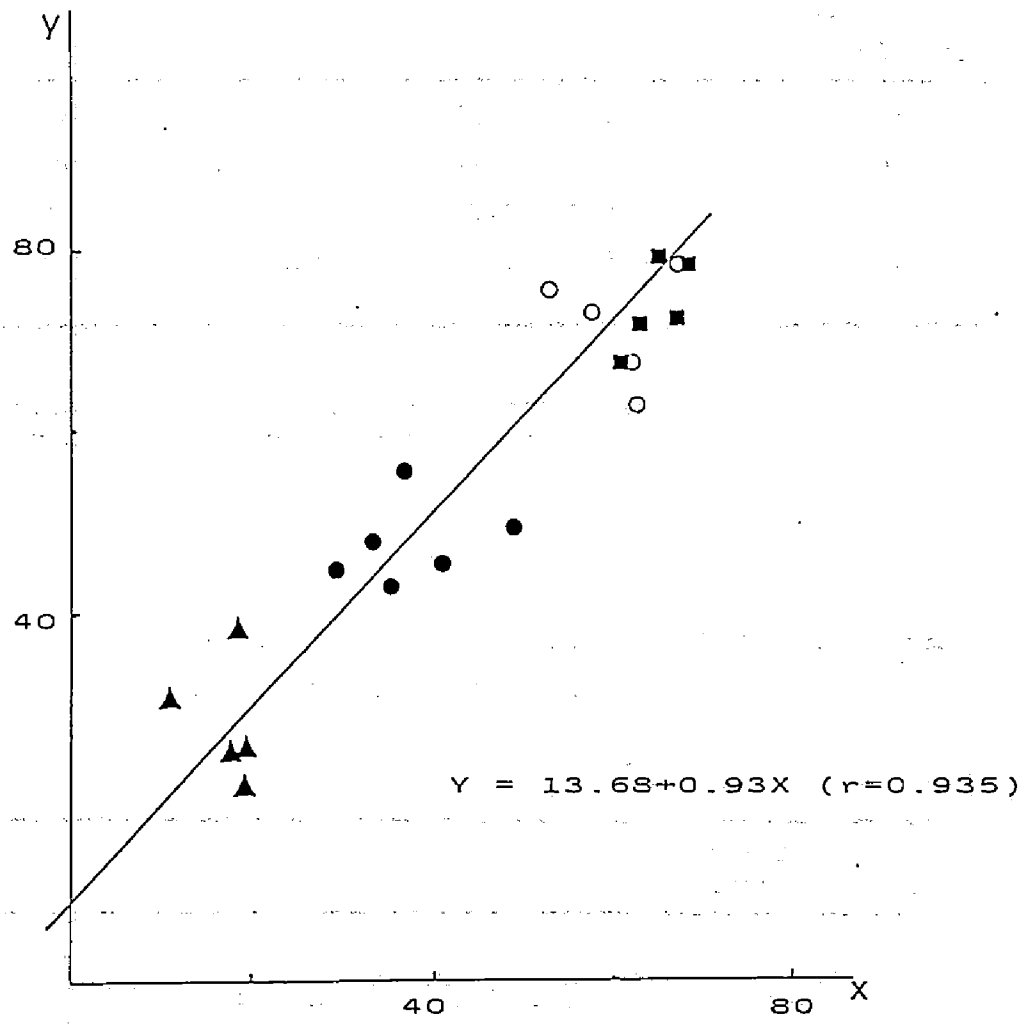
Approximately 3g of soybean cake, ground through a 3 mm sieve, were introduced into each bag. Five bags with a 50 µm pore size and 5 bags with a 100 µm pore size were fixed on a ferrous chain (30 cm long) which was connected to the plug of the rumen cannula. The bags were introduced into the rumen just before the morning feeding and incubated for 2,4,6,12, and 24 hours. At the end of the incubation the bags were removed from the rumen and washed at least four times with cold water by means of a simple manually operated washing apparatus. They were then squeezed

gently by hand and dried at 80°C for 48 hours. The dried bags were weighed to determine the amount of residual dry matter. The residue was ground and its nitrogen content was determined by Kjeldhal method.

The degradation rate of soybean cake protein was estimated by fitting the results to the model of ØRSKOV and McDONALD (1979): $P(t)=a+b(1-e^{-ct})$. Estimation of the parameters a, b and c was made by FACOM-M 382 at the Data Processing Center of Kyoto University using its " SALS " package.

The significance of the difference between the means in each experiment was determined by analysis of variance.

Figure 2-2. Linear correlation of protein degradability between faunated and defaunated rumen contents



X: N degradability of protein source measured with defaunated rumen contents

Y: N degradability of protein source measured with faunated rumen contents

■ lupine seed, ○ peanut cake, ● soybean cake, ▲ fish meal

Table 2-1: Effect of protozoa on the *in vitro* degradability of various protein sources (% of degradable N in total N ; mean± s.e.)

source	DF	F	statistical significance
Lupine seed	60.7 ± 3.4	70.9 ± 2.4	P< 0.01
Peanut cake	60.2 ± 2.4	71.7 ± 2.8	P< 0.05
Soybean cake	37.2 ± 2.7	47.6 ± 1.9	P< 0.05
Fish meal	17.0 ± 1.4	27.9 ± 2.9	P< 0.05

DF : incubated with defaunated rumen contents,
F : incubated with faunated rumen contents

Table 2-2 : Effect of protozoa on the *in vitro* degradation rate of soluble protein (means ± s.e.)

source	DF	F	statistical significance
Azocasein (1)	0.82 ± 0.20	0.64 ± 0.09	P<0.05
Casein (2)	12.00 ± 1.22	8.86 ± 1.04	P<0.01

(1) mg Azocasein hydrolyzed / ml of rumen fluid / hour

(2) µg Ammonia produced from casein degradation / ml of rumen fluid / hour

DF : incubated with defaunated rumen contents

F : incubated with faunated rumen contents

RESULTS

Faunated sheep had $5x10^5$ protozoa/ ml in their rumen. The population consisted of Entodinium sp.(80-90% of population), Polyplastron multivesiculatum (10-20%), and holotrichs(10-20%). The presence of a small number of Enoploplastron was observed in some sheep. Defaunated sheep had no protozoal contamination throughout the experiment.

The effect of protozoa on the in vitro protein degradabilities of lupine seed, peanut cake, soybean cake and fish meal is shown in Table 2-1. The values obtained with defaunated rumen contents were always lower than those from faunated rumen contents. The difference in protein degradability between the two animal types was an absolute constant for all tested protein sources (approximately 10 points). In relative values(DF/F) the presence of protozoa had a larger effect on proteins whose degradability was low. A linear correlation was observed between in vitro degradabilities measured with faunated rumen contents(y), and defaunated rumen contents(x): $Y=13.68+0.93X$ ($r= 0.935$) (Figure 2-2).

The degradation rate of azocasein and casein measured with in vitro method were significantly lower in faunated compared to defaunated sheep : 0.64 ± 0.03 vs.0.82 ± 0.06 mg azocasein hydrolyzed / ml of rumen contents / hour ($p < 0.05$),8.86 ± 1.04 vs.12.00 ± 1.22 µg ammonia produced from casein degradation / ml of rumen contents/ hour($p<0.01$)(Table 2-2).

Table 2-3. Effect of protozoa on protein degradability of soybean cake measured with " in sacco " method.

	D F		F			F vs.	50 vs.	Inter-
pore size(μm)	50	100	50	100	SEM	DF	100	action
a(%)	14.45	15.07	16.85	15.02	0.79	NS	NS	NS
b(%)	70.40	79.80	78.63	84.97	1.70	**	**	NS
c(% h^{-1})	8.71	9.16	8.75	10.40	0.22	*	**	*

DF: incubated in defaunated sheep rumen, F: incubated in faunated sheep rumen.
a,b,c,: parameters of $P(\%) = a + b(1-e^{-ct})$ ØRSKOV & McDONALD,1979.
Inter action: interaction between protozoa and pore size.
SEM: standard error of mean.
* $p < 0.05$, ** $p < 0.01$, NS: not significant

The effect of protozoa on the degradability of soybean cake protein, measured by the *in sacco* method, is shown in Table 2-3. No significant difference was observed between defaunated and faunated sheep in the "a" parameter which is considered to be the rapidly soluble fraction. Pore size also had no significant effect on this parameter. However, both the presence of protozoa and larger pore size significantly increased the "b" and "c" parameters. Both had a significant effect on "b" parameter($p<0.01$), and the pore size had a larger effect ($p<0.01$) than the protozoa ($p<0.05$) on parameter "c". The interaction of these two factors on the "c" parameter was also significant ($p<0.05$).

DISCUSSION

In vitro determinations

The small effect of protozoa on the degradation of highly soluble proteins observed in this experiment confirms the results obtained by ONODERA and KANDATSU (1970) and NUGEANT and MANGAN (1981). Protozoa, however, have a negative effect on the number of bacteria in the rumen; defaunation promotes 2-5 fold larger bacterial population compared to those in faunated sheep or calf rumen(e.g., EADIE and GILL, 1971, DEMEYER and VAN NEVEL, 1979). According to SIDDONS and PARADINE(1981), the primary factor which determines the degradation of soluble proteins is the size of the bacterial population, probably because soluble proteins can attach to the bacterial surface where protease and peptidase

Table 2-4. Effect of protozoa on the *in vitro* azocasein degradation (mg azocasein hydrolyzed / ml of rumen fluid or bacterial suspension / hour ; means± s.e.)

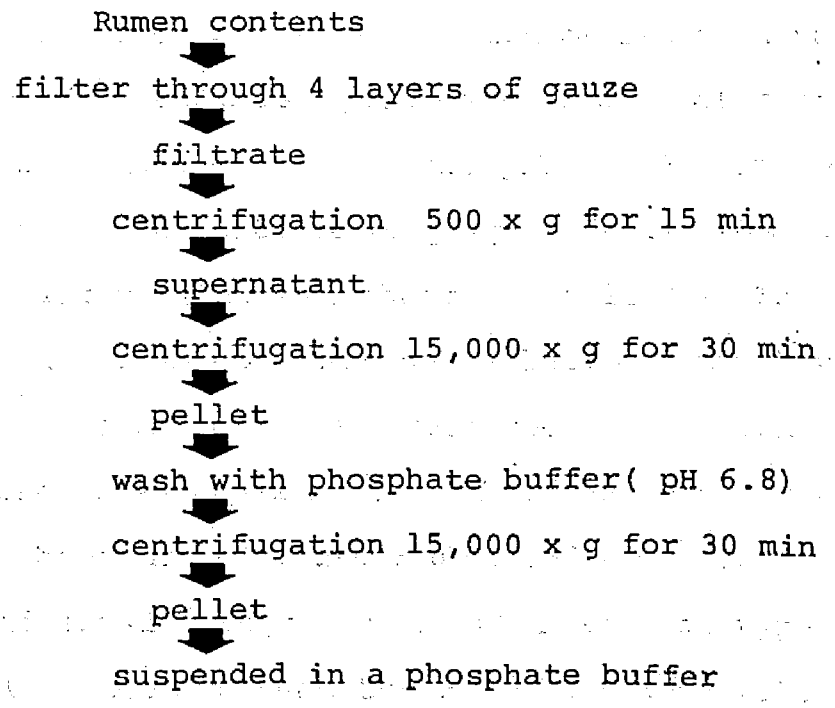
	DF	F	statistical significance
Filtered rumen fluid	1.174 ± 0.015	1.102 ± 0.018	P< 0.01
abs 3	0.141 ± 0.001	0.133 ± 0.003	P> 0.05
abs 2	0.051 ± 0.003	0.050 ± 0.001	P> 0.05

DF : inocula were taken from defaunated sheep
F : inocula were taken from faunated sheep

abs 3 : concentration of bacterial suspension was adjusted by spectrophotometer at 660 nm to 3.00.

abs 2 : concentration of bacterial suspension was adjusted by spectrophotometer at 660 nm to 2.00.

Diagram 2-1. Differencial centrifugation for bacterial suspension.



are located (NUGEANT and MANGAN 1981). Therefore, the degradation of soluble protein may be more intensive in the defaunated rumen than in the faunated. This hypothesis is supported by the fact that proteolytic activity was higher in defaunated sheep when it was measured using azocasein and casein which are totally soluble. It is probable that defaunation provokes the change of bacterial flora composition as discussed in chapters 1 and 4, and type of flora may affect soluble protein degradation. However ANNISON(1956) and WARNER (1956) demonstrated that change of flora composition induced by changes in dietary condition had no effect on the proteolytic activity of rumen contents. It is expected that changes in bacterial flora composition induced by defaunation may not have important effects on soluble protein degradation. Azocasein degradation rate was measured with bacterial suspension prepared from either defaunated or faunated rumen contents in order to confirm this hypothesis. Bacterial suspension was prepared by differential centrifugation(Diagram 2-1) using Suffolk ewes fed a concentrate:roughage = 50:50 diet mentioned in chapter 1. The results are shown in Table 2-4. In this experiment defaunated rumen contents had a larger activity of azocasein degradation than that of faunated rumen contents ($p<0.01$), while bacterial suspensions of which the concentration were controlled to absorbance 2.0 and 3.0 at 660 nm (slit 2 mm, path length 1 cm) had similar degradation rates in both types of animal. These results confirm that the rate of degradation of

soluble protein does not depend on type of bacterial flora, but on the number of bacteria. It is, therefore, certain that soluble protein is degraded more intensively in defaunated rumen than in faunated rumen.

In Sacco determinations

The amount of rapidly soluble protein, fraction "a", is a physical characteristic of each protein source. As with the in vitro results, we have shown that protozoal presence in the rumen has no effect on soluble proteins.

The insoluble potentially degradable protein, fraction "b", is due to the combined attacks of both bacteria and protozoa, which have completely different modes of degradation. Protozoa ingest entire particles of feed, while bacteria adhere to the surface of particle. Like KAYOULI et al. (1983),present results show that in the presence of protozoa, which causes a decrease in bacterial numbers, the degradability of fraction "b" and the rate of this degradation, parameter "c", are higher than in the defaunated rumen. Protozoal ingestion of dietary particles causes an increase in the relative enzyme concentartion in respect to the particle and more direct enzyme-substrate contact in the food vacuole. Protozoa, also, have a positive effect on the degradation of plant cell walls (JOUANY and SENAUD,1979b) which improves the accesibility of intracellular proteins to degradative enzymes. Cell wall degradation is often the limiting factor

in the proteolytic process in the rumen (GANEV et al., 1979). The increase in fraction "b" degradability seen when the pore size of the bags was increased is essentially due to an increase in physical loss from the bags. In effect, the measured differences between the 50 µm and 100 µm mesh size are equivalent, slightly larger in defaunated animals compared to those measured in faunated animals. Protozoa had no effect on the degradation rate "c" of fraction "b" in the 50 µm bags, however, there was a significant effect in the 100 µm bags. One can conclude from this observation that small Ophryoscolecidae(<50 µm) and also the Isotrichidae, the only ciliates capable of entering the 50 µm bags, have no effect on the rate of proteolysis in the rumen. It is also possible that this parameter could be more strongly influenced by the larger Ophryoscolecidae(100 µm). These conclusions are supported by the statistical significance of the interaction " protozoa-pore size".

ØRSKOV and McDONALD (1979) have proposed another model for the prediction of ruminal protein degradation: P(%)= a+(bc)/ (c+kr) with kr being the out flow rate of marked particles from the rumen. In the present experiment, the outflow of protein particles was not measured. However, KAYOULI et al.(1983) reported an increase in the rumen outflow rate of chromium mordanted soybean cake or lucerne hay following defaunation and same result was obtained in the experiment in chapter 4. These results suggest that insoluble protein particles stay in the rumen for a

Table 2-5. Effect of protozoa on the prediction of protein degradability of soybean cake

	Kr(h^{-1})	Predicted degradability(%)	
		50 μ	100 μ
DF	0.065	54.7	61.6
F	0.054	65.3	70.7

Kr: out flow rate of chromium mordanted lucerne hay pellets (details are shown in Table 4-8)

DF: defaunated sheep, F: faunated sheep. 50 μ,100 μ: pore size of nylon bag. Predicted degradability calculated according to the following formula; P(%)= a+(bc)/(c+Kr) (ØRSKOV & McDONALD, 1979)

shorter time and thus are exposed to less intensive proteolysis when protozoa are removed from the rumen. Table 2-5 shows the estimated protein degradability according to this model, using the kr value taken from the data appeared in chapter 4. In these conditions, the protein degradability in the defaunated rumen is approximately 85% of that in the faunated rumen independent of the nylon bag's pore size.

Present experiment suggests that protozoal effect on the protein degradability is more evident when the dietary proteins are not readily soluble.

SUMMARY

In this experiment, role of protozoa on rumen protein degradation was studied using 5 defaunated and 6 faunated sheep. Rumen contents taken from these sheep were used as the inocula for the determination of in vitro protein degradability of four protein sources which have different protein solubilities. Same rumen contents were used for the determination of degradation rate of totally soluble protein, azocasein and casein. In sacco determinations were also done using 3 defaunated and 3 faunated sheep to measure the degradation rate of soybean cake protein. In this measurement, two different pore sizes (50 µm, 100 µm) were used. The results were analyzed by the model of ØRSKOV and McDONALD (1979) ; $P= a + b(1 - e^{-ct})$.

The in vitro degradability of proteins were significantly decreased in defaunated sheep compared to the faunated. The protozoal effect was more important when protein solubility was low (DF/F value with lupine was 0.85, 0.84 with peanut cake, 0.78 with soybean cake, 0.61 with fish meal). Azocasein and casein degradation rates were significantly higher with defaunated sheep rumen contents than with that of faunated.

The in sacco study showed that neither protozoa, nor the pore size of bags had any action on the degradation of protein fraction immediately soluble in rumen fluid (parameter "a"). While both factors increased the insoluble, potentially degradable fraction (parameter "b") and its degradation rate (parameter

"c"). Protozoal effect was clearer with 100 µm bags. The significant interaction between protozoa and pore size on parameter "c" was observed. It is suggested that large Ophryoscolecidae (> 50 µm) influence more strongly on insoluble protein degradation than small species (< 50 µm).

It is confirmed that protozoa do not have the dominant effect on soluble protein degradation and that protozoa, particularly the large Ophryoscolecidae, increase insoluble protein degradation. These results suggest that protozoa limit the passage of dietary protein into the small intestine.

CHAPTER 3. UTILIZATION OF NUCLEIC ACID PURINE BASES AS A MARKER FOR RUMEN MICROBIAL PROTEIN SYNTHESIS

INTRODUCTION

It is necessary to estimate both protozoal and bacterial protein synthesis in the rumen in order to study the protozoal contribution to the protein digestion in the ruminant. Rumen microbial protein synthesis is usually estimated by a marker method in which a specific substance contained in microorganisms or isotopic elements incorporated in microorganisms is measured as a microbial marker. It is necessary that the marker is not contained in the diet, or completely degraded in the rumen if it is contained in the diet. It is also necessary that the marker is neither degraded, nor absorbed, nor produced between the reticulum and the proximal duodenum where the cannula is usually placed.

2-6 diaminopimeric acid (DAP)is a specific amino acid which composes the mucopeptide component in the cell wall of gram-negative bacteria. Since this amino acid is absent in the plant or animal tissues(MASON and BECH-ANDERSEN,1976), DAP is widely used as a bacterial marker in many works. However the concentration of DAP depends on the bacterial species (WORK and DEWEY,1953), and this marker allows, naturally, to estimate only the bacterial protein synthesis. Ribonucleic acid(RNA) is also an usual microbial marker. The utilization of RNA is based on the positive correlation between the cellular protein synthesis and the concentration of RNA. This correlation was demonstrated in some bacterial species which are not rumen habitats(MITCHELL and

MOYDE,1951; GALE and FOLKES,1953;JEENER,1953) and some rumen bacteria (ELLIS and PFANDER,1965). The ratio of RNA-N to total-N in the rumen bacteria vary from 0.14 to 0.25 and from 0.08 to 0.15 according to SMITH (1969) and to CZERKAWSKI (1976), respectively. For same ratio in the rumen protozoa, 0.11-0.12 in the Entodinium species (GAUSSÈRES and FAUCONNEAU,1966) and 0.07-0.15 in the mixed rumen protozoa (CZERKAWSKI,1976) were reported. Because of the similarity in this ratio, RNA allows to estimate total microbial (protozoa + bacteria) protein synthesis. However the contribution of the microbial protein to non ammonia nitrogen (NAN) entering the duodenum estimated by RNA method is generally larger than those estimated by other marker methods (HARRISON and McALLAN,1980). This phenomenon is explained by the presence of dietary RNA which escapes from the rumen degradation (SMITH et al.,1978).

Deoxyribonucleic acid (DNA) can be used as the microbial marker with the same principle, but the concentration of DNA in the microorganisms is much more inferior to that of RNA. Therefore DNA is not considered as a suitable marker.

^{35}S,^{32}P and ^{15}N are used in the isotopic marker method; a solution of the inorganic salt of these isotopes is infused into the rumen and rumen microorganisms uptake these salts to make their cellular components. For ^{35}S , a solution of $Na_2{}^{35}SO_4$ is infused into the rumen and the bacteria synthesizes its sulfur amino acid in metabolizing the $^{35}SO_4{}^{2-}$. ^{35}S can also be incorpo-

rated into the protozoal protein because the protozoa ingest the bacteria whose protein is already marked by ^{35}S. Therefore, there is a lag-time between bacteria and protozoa for the time needed to achieve the plateau values of specific activity.

BEEVER et al.(1974) demonstrated that the specific activity of methionin ^{35}S in the protozoa reaches to a similar level to that observed in the bacteria after 16 hours infusion of $Na_2{}^{35}SO_4$ solution (0.225 μCi/ml). This isotope can be utilized as a microbial marker.

^{32}P is also used in the estimation of microbial protein synthesis. The principle of this marker method is same as ^{35}S; the protozoa and the bacteria uptake $^{32}PO_4{}^{3-}$ to synthesize the phospholipids and nucleic acids. However the time needed to achieve the plateau value of specific activity both in bacteria and in protozoa is much longer than that of ^{35}S (JOHN and ULYATT, 1984). Moreover the energy of ray emit by this radioisotope is strong (ten times superior to that of ^{35}S), the aplication of this isotope to the in vivo trial generally limited.

^{15}N can be used as the microbial marker with the same manner as ^{35}S or ^{32}P. This isotope is not radioactive, but its determination needs the high cost analytical apparatus.

For the specific marker of protozoa, 2-aminoethylphosphonic acid(AEP) and phosphatidyl-choline(PC) were used.(ABOU AKKADA et al.,1976; JOHN and ULYATT,1984). However the presence of AEP was detected in many diet and also in certain bacteria(LING and

BUTTERY,1978), consequently the validity of this marker is questioned.

Although ^{35}S seems to give the most correct result among these markers (LING and BUTTERY,1978; SIDDONS et al.,1982; COCKBURN and WILLIAMS,1984; WHITELAW et al.,1984), the reliability of these markers is still under discussion.

The objective of this chapter is to study the new marker method, proposed by ZINN and OWENS (1980), in which nucleic acid purine base is determined by a relatively simple procedure.

Utilization of the nucleic acid as a microbial marker was first suggested by GAUSSÈRES and FAUCONNEAU (1966). A series of studies on the utilization of this marker were completed by McALLAN and SMITH (1969,1972,1973,1974,1983,1984), in which these authors reported the advantages and disadvantages of this method. The analytical procedure for determining nucleic acid is so long and laborious that it is difficult to apply this method to trials which provide large quantities of samples. Certain workers use a simpler analytical procedure (TOPPS and ELLIOT ,1965) or determine nucleic acid purine base (McDONALD, 1954). However, McALLAN and SMITH (1969) pointed out that these analytical methods are less reliable due to the presence of interfering substances. In 1980, ZINN and OWENS proposed an analytical procedure for nucleic acid purine bases which seems to be both simple and efficient. It consists of an oxidating hydrolysis to liberate the bases, then to precipitate them by forming a silver-purines

complex at a pH between 2 and 3. The precipitate, washed to eliminate pyrimidine bases, is solubilized at a pH below 2 with heating. The optical density of purine bases is read at 260 nm. Equivalents of yeast RNA (RNA eq) is determined using yeast RNA as the RNA standard. The procedure, however, was published only in the form of a simple diagram which did not provide the information necessary to use it. This study was then conducted to establish an analytical method for purine bases, based on the diagram of ZINN and OWENS, in which the efficiency of oxidative hydrolyzation of nucleic acid, liberation of purine bases and their precipitation as silver salts were all verified by means of HPLC. The best procedure for sample treatment prior to analysis for bacteria and duodenal digesta were also investigated. The daily flow of bacterial nitrogen at proximal duodenum of defaunated sheep was finally estimated using nitrogen-purine base ratio in rumen bacteria and the daily flow of purine base. These estimates were then compared with those given by DAP and ^{35}S which were considered to be reference values that allowed the selection of the best method of sample treatment and preservation for purine base analysis in regards to estimating rumen microbial synthesis. The three methods (DAP, ^{35}S and purine bases,PB) were compared in defaunated animals to eliminate possible interference from protozoa.

Table 3-1. Diet composition (% on air dry basis)

Ingredient	L	S
lucerne hay	65.0	-
NaOH treated wheat straw	-	66.8
barley	30.0	-
sugar beet pulp	-	14.2
soybean cake	-	5.2
peanut cake	-	8.6
urea	-	0.85
wheat straw	2.7	-
vitamin mineral supplement(1)	-	1.95
Cr-mordanted lucerne hay(2)	2.3	2.4
OM (% in DM)	92.1	89.8
N (% in DM)	2.0	1.9
ME (Mcal/kg DM)	2.35	1.55
predicted N solubility(3)	0.25	0.43

(1) $Ca(H_2PO_4)_2$:55%, NaCl:26%, S: 1%, Mg_2SO_4:9%, Na_2SO_4:7%; trace elements:2%; Vitamin A(50000 IU/g):27g/100kg, Vitamin D_3(100,000 IU/g):5g/100kg, Vitamin E(250 IU/g): 100g/100kg.

(2) UDEN et al.(1980)

(3) INRA, Alimentation des Ruminants.pp485-486.(1978)

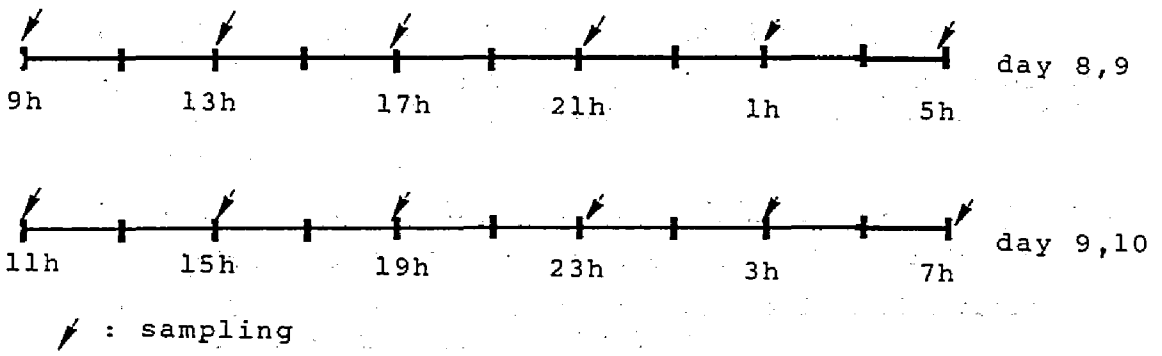
MATERIALS AND METHODS

Animals and Diets

Six adult wether sheep (race: Texel, body weight:60 Kg) fitted with rumen and simple T-shaped duodenal cannulae were used in this study. The sheep were defaunated by above mentioned method two months before the experiment. Three sheep received a diet (L) which was composed of lucerne hay, barley, and wheat straw. The other sheep received a diet (S) which was composed of NaOH treated wheat straw, sugar beet pulp, soya bean cake, peanut cake meal and urea. Detail of each diet is given in Table 3-1. Both diets were pelleted and distributed (150 g) at three hours intervals by means of an automatic feeder. Water and mineral licks were always available to the sheep. A solution of $Na_2{}^{35}SO_4$ was infused into the rumen via a rumen cannula at the rate of 250 µCi per animal per day and the infusion was started three days before the duodenal sampling. Chromium(Cr^{3+}) mordanted lucerne hay (UDEN et al., 1980) was incorporated into the diet and distributed to the sheep at the rate of 2g Cr per day. The distribution was started a week before the duodenal sampling. 70 ml of duodenal contents was taken every 4 hours during two consecutive days according to the sampling program shown in Figure 3-1. All the samples were then pooled and preserved at -15°C until the analysis. 400 ml of rumen contents were taken every 2 hours for 10 hours on the following day. Bacterial samples were obtained by

differential centrifugation as described by JOUANY and THIVEND (1972).

Figure 3-1. Sampling schedule of duodenal digesta.



Analytical procedure

For the nucleic acid purine base determination, two methods were used : (1) the spectrophotometric method was carried out to determine the concentration of equivalents of yeast RNA (RNA eq) in the different samples of bacteria and digesta (Diagram 3-1). This part of the experiment was used to determine some aspects of method which were not described in the original paper of ZINN and OWENS (1980); i.e. filter pore size, speed and time of centrifugation, elimination of interfering substances. (2) a HPLC study was carried out using the method described by SCHWEINSBERG and TI LI LOO (1980) for the analysis of nucleotides which was modified to analyze purine bases

Diagram 3-1. Method of purine base analysis for rumen bacteria and intestinal digesta.

Step 1) Weight 0.5 g of freeze-dried sample into 30 ml test tube with screw cap

2) Add 3 ml of 70 % $HClO_4$ and hydrolyse for 1 hour at 95°C (in a water bath)

3) Add 17.5 ml of 0.0285 M $NH_4H_2PO_4$ and mixed throughly and then place in 95°C water bath for an additional 15 min.

4) Filter hydrolysate through a grass filter (Pylex No.5)

5) Mix 0.5 ml of filtrate, 0.5 ml of 0.4 M $AgNO_3$ and 9 ml of 0.2 M $NH_4H_2PO_4$ in a centrifuge tube. Allow to stand for 30 min.

6) Centrifuge at 25,000 x g for 15 min.

7) Wash pellet with 10 ml of 0.2 M $NH_4H_2PO_4$ which was adjusted to pH 2 with conc. H_2SO_4 and centrifuge at 25,000 x g for 15 min.

8) Add 10 ml of 0.5 N HCl to the pellet and mixed thoroughly Place in 95°C water bath for 30 min.

9) Filter with a coarse filter paper

10) Read at 260 nm.

The purine base standard curve is established using yeast RNA (60 mg) which has undergone all these steps. The filtrate of step 9 is succesively diluted by 0.5 N HCl.

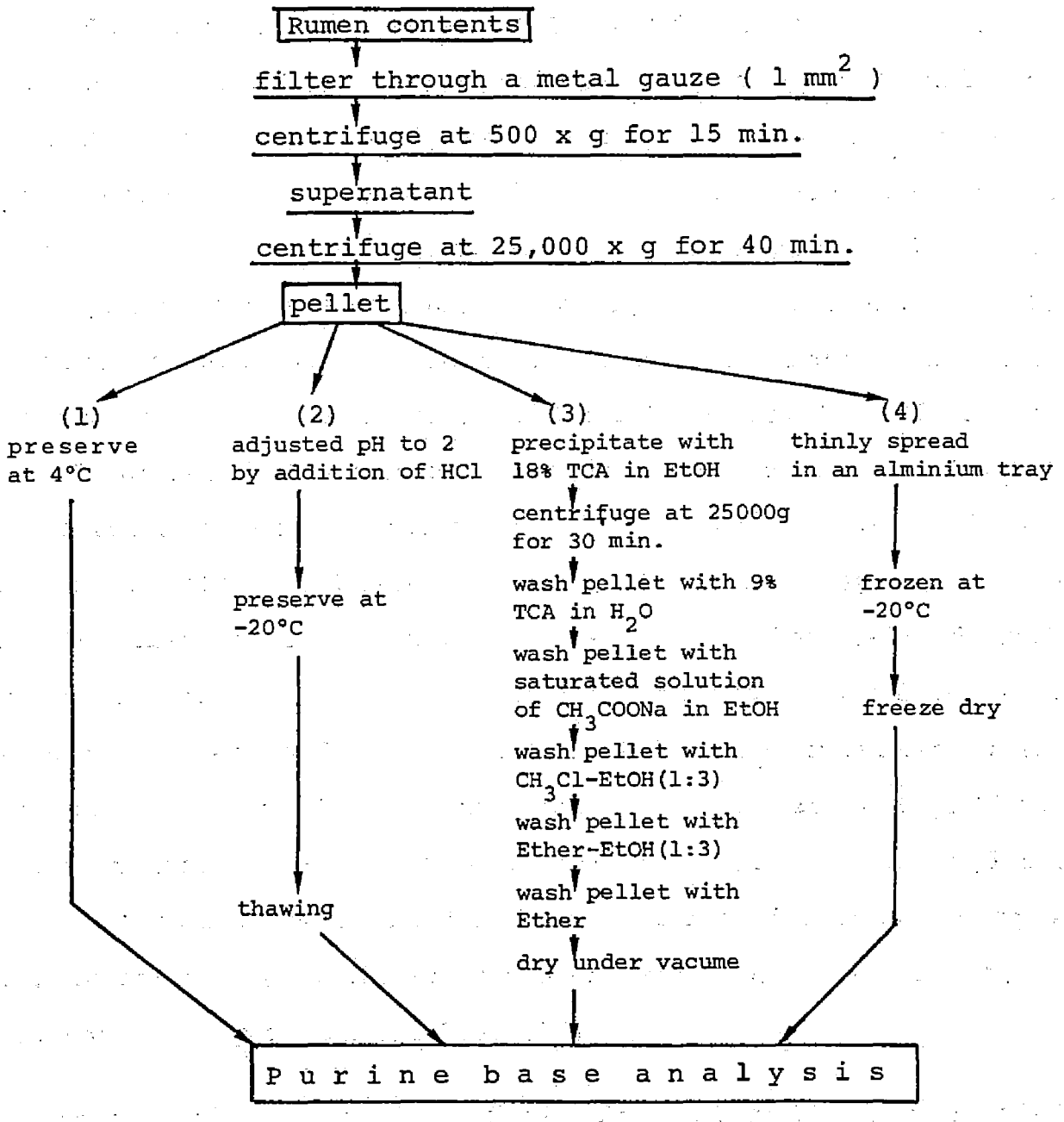
liberated from nucleic acid by perchloric acid hydrolyzation. All the instruments, which were composed of a SP 8700 solvent delivery system, a SP 8750 pumping unit, a SP 8440 UV/VIS detector and a SP 4270 integrator, was obtained from Spectra Physics (San Jose. Ca.USA). This system was equipped with RP-18 OD-224 column (Brownlee labs. Santa Clara.Ca.USA). A phosphate buffer was made of 0.06 M K_2HPO_4 and 0.04 M KH_2PO_4 and was adjusted the pH to 6 by phospholic acid. This buffer was used as a basal solution for the mobile phase. The solvent gradient was acheived by increasing the concentration of pure methanol from 10% to 20% in the mobile phase for 15 minutes and then withdrawing it immediately. The solution was pumped at the rate of 1 ml/min. All the solution, including the samples, was ultrafiltered through a milipore 0.1 µm filter prior to the usage. Purine bases were analyzed by this system 24 hours after RNA eq determinations. Solutions injected into the chromatograph were first brought to pH 12 with 12 N NaOH to solubilize those purine bases (mainly guanine) which precipitate at pHs less than 2 after standing overnight. This experiment served to verify the efficiency of the nucleic acid hydrolyzation, the efficiency of purine base-silver salts complexation, and the recovery rate of purine bases without the pyrimidine bases contamination.

DM was calculated after drying at 80°C for 48 hours. Total nitrogen was determined by the kjeldhal method. Chromic oxide was determined by a modified method of MATHIESON (1970). ^{35}S was

determined principally by the method of MATHERS and MILLER (1980); 300-500 mg of freeze-dried sample was oxydized by 20 ml of performic acid(18 ml HCOOH + 2 ml H_2O_2)for 12 hours. The oxydation was stopped by the addition of bromhydric acid. Then 20 ml of HCl(6 N) was added and placed in the oil bath at 120°C for 24 hours. After hydrolyzation, HCl was totally evaporated by means of a rotaly evaporator and all the amino acid was recuperated into the citrate buffer(pH 3.2). This solution was filtered through a glass filter (Pylex No.4)and the filtrate was separated into two portions; one for the ^{35}S determination, and another for DAP determination. 1 ml of saturated $BaCl_2$ was added to 7 ml of filtrate and the solution was centrifuged at 2000 x g for the elimination of free anion of $^{35}SO_4$. 1 ml of supernatant , 4 ml of distilled water and 10 ml of scintillation fluid (INSTA-GEL, Packard Instrument Corp. USA) were introduced into a scintillation bial and well mixed. Finally, the specific activity of ^{35}S was measured with a TRICARB 460 CD spectrometer (Packard Instrument Corp. USA).

DAP determination was conducted as described by COLLOMBIER(1981). 6 ml of filtrate was analyzed by a semi automated liquid chromatography using ion exchange resine (DOWEX 50 W-X8). Behind the column , the amino acids were reacted with ninhydrin acid at pH 1 and measured with a spectrophotometer(VITATRON , Malakoff, France)at 420 nm. Under such an acidic condition, only DAP and proline make the yellow color.

Diagram 3-2. Sample treatments



Sample treatment and preservation prior to analysis

According to McALLAN and SMITH (1969, 1983), sample treatment and preservation have an important effect on RNA analysis, since the concentration of RNA-N in bacterial preparations can change from 9.89 mg/ g DM to 4.02 mg/ g DM according to the method of sample preservation. Based on these published results and on the hypothesis of these authors, each sample of bacteria was devided into four portions with each one allotted to one of four treatments :(1) storage at 4°C for 72 hours, (2) addition of 37 % of HCl until pH 2 was obtained and storage at -20°C,(3) fat extraction according to the method of McALLAN and SMITH (1969) (2nd to 6th step in their method), (4) thinly spread in an alminium tray and frozen rapidly at -20°C,then freeze-dried. Each sample of duodenal digesta was divided into three portions after being thawed and allotted to one of following treatments; (5) directly analyzed, (6) fat extraction, (7) freeze drying. These sample treatments are shown in Diagram 3-2. For the wet samples (1,2,5), 3 g of the bacterial sample and 5 g of duodenal sample were hydrolyzed with perchloric acid, while 0.5 g of sample was used for the dry samples (3,4,6,7).

Comparison of different methods for determining microbial synthesis

Bacterial nitrogen flow at duodenum was calculated using the three different marker methods in defaunated sheep. The

utilization of defaunated sheep allowed the comparison of these marker methods.

All the results was statistically analyzed by analysis of variance (SNEDECOR and COCHRAN 1967).

RESULTS AND DISCUSSIONS

Alterations in the original method of ZINN and OWENS

The conditions for filtration (step 4) and centrifugation (step 6 and 7) were indicated and one other step (step 9) was added in Diagram 3-1.

Step 4 : different types of filters and different pore sizes were tested. Filtration using glass filter n 5 (Pylex) with vacuum eliminated all black particles present in the liquid after hydrolyzation. Perchloric acid, however, reacts with the glass filters, which must be changed every 100 samples.

Step 6 and 7 : the recovery rate of the Ag-purines complex by centrifugation must be quantitative. Our study showed that this condition was reached with 25,000 x g for 15 minutes.

Step 9 : many fine particles (probably chlorides) are present in the liquid containing free purine bases when the Ag-purines complex was dissolved in step 8. These particles sometimes interfere with spectrophotometric determinations on some duodenal samples, giving incorrect values for RNA eq (50 % too high). A filtration with coarse paper (step 9) eliminated interference, which is why this step was added for all the samples. Under the conditions used, the method gives reproducible results (on microbial nitrogen basis) equal to those obtained with ^{35}S and DAP.

Table 3-2 : Recovery rate of adenine and guanine determined by HPLC[1]

	weight of additional purine base (mg)	recovery rate of purine base (%)[2]
Adenine	8.0	101.1 ± 2.7
Guanine	3.0	101.6 ± 2.7
Bacteria + adenine	8.0	90.0 ± 1.9
Bacteria + guanine	3.0	90.6 ± 1.9
Bacteria + yeast RNA	45.0	96.4 ± 11.1

1) The solution of step 10 (diagram 3-1), which was analyzed by HPLC, was adjusted to pH 12 by 12 N NaOH prior to analysis.

2) Means ± s.d.

Verification of the validity of ZINN and OWENS method by HPLC
(Table 3-2)

When pure adenine or guanine was analyzed as shown in Diagram 3-1, the recoveries of adenine and guanine were 101.1 % and 101.6 %, respectively. When each purine base was added to a bacterial sample, the recoveries of adenine and guaine were 90.0 % and 90.6 %, respectively. Under the same conditions, the recovery of purine bases in yeast RNA added to bacterial samples was 96.4 %. These results suggest that the oxidative hydrolyzation of nucleic acid is complete and that precipitation of purine bases as a silver salt and its solubilization are also complete. The HPLC measurements allow to conclude that pyrimidine bases were totally eliminated at step 10 after washing and centrifugation of precipitate (step 7 in Diagram 3-1). Finally, it is shown that adenine / guanine ratio determined in bacteria isolated from 11 different rumen samples taken from sheep (1.41 $\pm$ 0.08) was not significantly different from the same ratio measured in yeast RNA (1.32 $\pm$ 0.03). This result shows that purine base composition in yeast RNA, which is used as standard, correspond to that of rumen bacteria. After verifying the validity of the RNA eq analysis, we measured the influence of sample treatment and preservation on microbial synthesis estimated with this method.

Sample treatment and preservation

Sample treatment and preservation have a significant

Table 3-3. Effect of sample treatment prior to analysis on the purine base content of bacterial preparations

	diet	wet sample		dry sample		SEM[4]	statistical significance[5] of		
		stored at 4°C	stored at -20°C[3]	fat-extracted	freeze-dried		treatment	diet	int.
Purine base content (1)	S	56.65[a][6]	82.74[b]	88.88[b]	88.95[b]	4.25	*	*	NS
	L	46.38[a]	67.95[b]	73.77[b]	73.04[b]	3.30			
Purine base : N ratio (2)	S	0.70[a]	1.02[b]	1.10[b]	1.09[b]	0.05	*	*	NS
	L	0.59[a]	0.86[b]	0.93[b]	0.92[b]	0.04			

1) expressed as mg yeast RNA / g of DM of bacterial preparation

2) expressed as mg yeast RNA / mg total nitrogen of bacterial preparation

3) pH of bacterial preparations was adjusted to 2 by HCl prior to freezing

4) standard error of means

5) analysis of variance on the effects of treatment, diet and interaction of these two factors on purine base content and purine base : N ratio; * $p<0.05$, NS: not significant

6) means within a row followed by different letters differ significantly($p<0.05$)

Table 3-4. Effect of sample treatment prior to analysis on purine base content of duodenal digesta[1]

	wet sample	dry sample			statistical significance[3] of		
diet	stored at -20°C	fat-extracted	freeze-dried	sem[2]	treatment	diet	interaction
S	33.65[a(4)]	13.28[b]	21.18[c]	3.47			
					* *	*	NS
L	36.20[a]	10.25[b]	19.73[c]	3.41			

1) expressed as mg yeast RNA / g of DM of duodenal digesta

2) standard error of means

3) analysis of variance on the effects of treatment, diet and interaction of these two factors on purine base content ; * $p<0.05$, ** $p<0.01$, NS: not significant

4) means within a row followed by different letters differ significantly ($p<0.05$)

Table 3-5. Bacterial protein synthesis estimated by different methods in defaunated sheep and effect of sample treatment on purine base method.

	Diet (1)	^{35}S	DAP	Purine base			SEM (2)
		freeze-dried	dried	freeze-dried	fat-extracted	frozen (wet)	
Bacterial nitrogen flow at duodenum (g/d)	S	18.51[a]	20.55[a]	16.24[a]	10.72[b]	30.78[c]	1.95
	L	14.38[a]	15.64[a]	15.42[a]	10.86[b]	26.35[c]	1.49
Percentage of bacterial nitrogen in duodenal NAN(%)	S	64.2 [a]	71.4 [b]	56.3 [a]	37.4 [c]	105.6 [d]	6.3
	L	46.7 [a]	50.9 [a]	49.9 [a]	35.1 [b]	85.3 [c]	4.9

(1) Details are shown in Table 3-1

(2) Standard error of mean

(3) Values within a row followed by different superscript differ significantly($p<0.05$)

effect on purine bases concentration. Purine bases concentration in freeze-dried and fat-extracted samples of bacteria were the highest among the four treatments (Table 3-3). Purine base losses, approximately 10 %, were observed when bacterial samples were frozen and stored at -20°C for 14 days, this difference was not significant however. The losses reached an important level, 37 %, when bacterial samples were stored at 4°C for 72 hours. Like bacterial samples, the method of treatment and preservation of duodenal samples had an important effect on the results of purine base measurements. The concentrations of purine bases (RNA eq) in freeze-died digesta or in direct determined samples were respectively 2 and 3 times higher than those in fat-extracted digesta samples (Table 3-4). The determination of purine bases was not done on digesta samples stored at 4°C, because 4°C storage was shown to produce large losses in bacterial samples. The selection of most suitable method of preservation for both bacterial and digesta samples was determined by comparing the estimates of bacterial nitrogen flow at duodenum which were obtained by the three different markers (RNA eq, ^{35}S and DAP)(Table 3-5). Considering the results taken from ^{35}S or DAP measurements as the reference values, it appears that the samples of bacteria and duodenal digesta should be stored in the freeze-dried form, since the differences observed between the different marker methods were not significant. The bacterial nitrogen flow determined from RNA eq

on fat-extracted or frozen samples, however, was always significantly different from three other tested methods (^{35}S, DAP and RNA eq on freeze-dried samples). Fat-extraction lowered the bactrial nitrogen flow , whilst direct determination gave excessively high values. The abnormal values obtained with the wet duodenal or bacterial samples could be due to several factors : water contained in the wet samples diluted the perchloric acid (7 N), which was used for nucleic acid hydrolyzation, to 2.7 N in the wet digesta and to 5.8 N in the wet bacterial samples. Moreover, the samples which were hydrolyzed in this study were rich in organic matter which would utilize an unknown proportion of the added perchrolic acid for its digestion. These losses limit the amount of perchrolic acid which was truly available for nucleic acid hydrolyzation in the wet samples, although LORING (1955) reported that 1 N acid is sufficient to liberate all the purine bases in nucleic acid. The water content also caused an increase in the volume of hydrolysate at step 4 and thus decreased the concentration of purine bases in the aliquot (0.5 ml) taken at step 5. The abnormally high values obtained with wet duodenal digesta could be explained by the presence of substances which have a strong absorbance at 260 nm. The identification of these substances was not done in this experiment.The low values of purine base concentration observed in the fat-extracted duodenal digesta could be explained by a partial extraction of purine bases liberated during storage by the organic solvents used (

ethanol and ether) in steps 1 to 6 in the SMITH and McALLAN method (1969). The method proposed in Diagram 3-1, with some modifications of the original method of ZINN and OWENS (1980), can be used for the determination of RNA eq in the rumen bacteria and duodenal digesta. This method gives estimates of rumen bacterial nitrogen flow at the duodenum which are equivalent to those obtained using the more traditional markers such as ^{35}S or DAP. The use of RNA for estimating the microbial synthesis in the rumen is not valid, however, when dietary RNA flows into the duodenum, and this purine base method is not free from this problem as pointed out by HA and KEENELLY(1984). It is, therefore ,still desirable to adopt multiple utilization of microbial markers and validity of PB method should be further examined under different experimental conditions.

SUMMARY

Nucleic acid purine bases content was determined in bacteria and duodenal digesta taken from six defaunated wether sheep. This microbial marker method was, first, proposed by ZINN and OWENS(1980). This method, however, was published only in the form of a simple diagram which did not provide sufficient informations to use it. Moreover, recent investigation (McALLAN and SMITH,1983) indicates that mode of sample preservation has an important effect on RNA analysis. Therefore, this experiment was carried out for the determination of assay parameters for nucleic acid purine bases analysis.

Some modifications of original method were achieved and validity of the modified method was verified by HPLC. Recoveries of purine bases and yeast RNA were almost complete and these results indicate the validity of this modified method.

For the evaluation of effect of sample treatment on purine bases analysis, the sample were treated by different ways. The bacterial sample were treated as follows ; 1) stored at 4°C for 72 hours, 2) addition of HCl until pH 2 was obtained and storage at - 20° C, 3) fat extraction, 4) thinly spread in an alminium tray and frozen rapidly at - 20°C, then freeze-dried. The duodenal digesta were divided into three portions after being thawed and allotted to one of following treatments ; 1) directly analyzed, 2) fat extraction, 3) freeze-drying.

The selection of most suitable method of preservation for

both bacteria and duodenal digesta samples was determined by comparing the estimates of bacterial nitrogen flow at duodenum which were obtained three different markers (DAP, ^{35}S, PB). These results suggest that most suitable method of sample preservation is freeze-drying and that nucleic acid purine base method is reliable under normal feeding conditions.

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CHAPTER 4. ROLE OF PROTOZOA ON RUMINANT DIGESTION

INTRODUCTION

In chapter 2, it is demonstrated that soluble protein degradation depends on the bacteria rather than protozoa, while protozoa ,compared to bacteria, play an important role on the insoluble protein degradation. In this chapter, an in vivo trial was carried out using defaunated and faunated sheep fed two isonitrogenous diets which had different characteristics in solubility to elucidate mainly protozoal contribution to the nitrogen digestion. The effect of protozoa on ruminant digestion has been rarely studied in the in vivo trial(LINDSAY and HOGAN,1972), because of the difficulties in the estimation of digesta flow and also in the techniques of defaunation. In a recent decade, an accurate method for the estimation of digesta flow was established(FAICHNEY,1975) and a potent method of defaunation was established (JOUANY and SENAUD,1979a). This experiment was, then, based on these technical progress. Simultaneous utilization of different microbial markers in defaunated sheep allowed to confirm the reliability of these methods and also allowed to estimate the contribution of protozoal protein to the duodenal protein by difference between the values estimated with DAP method and those of PB or ^{35}S in the faunated sheep.

MATERIALS AND METHODS

Animals and Diets

6 Texel 1-2 years old wethers, weighing 60 Kg of body weight, were used. Each sheep was fitted with a rumen and a

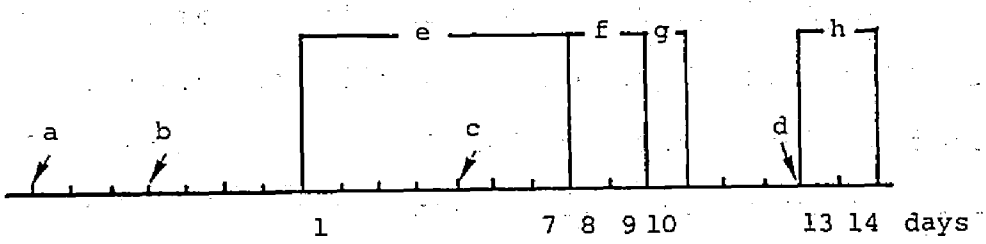
simple "T" shape duodenal cannula. The sheep were defaunated as described in chapter 2 at two months before the start of the experiment. The experiment was consisted with two consecutive periods; defaunated and faunated period. At the end of defaunated period, the sheep were inoculated with rumen contents which contained rumen protozoa taken from the sheep fed various diets.

Two isonitrogenous diets were used; one diet (L), which favoured a large protozoal population and which was also high in poorly degradable protein, was mainly composed of lucerne hay, while another diet (S), which did not promote a large rumen protozoal population but which was rich in highly degradable protein, was mainly composed of NaOH-treated wheat straw. The detail of these diets is shown in Table 3-1. Both diets were pelleted and distributed in eight equal portions at three hour intervals by means of an automatic feeder. Three sheep were fed (L) diet and three others were fed (S) diet for the first trial in each period. The diets were then interchanged and the trial repeated. Three weeks were allowed between the trials as an adaptation period. Water and mineral block were always available to the sheep.

Experimental procedure

Chromium oxide (Cr) mordanted lucerne hay (UDEN et al., 1980) was incorporated into the pelleted diet and distributed at the rate of 2g Cr per day per animal. A solution of polyethylene glycol (PEG)-4000 was infused via a rumen cannula at the rate

Figure 4-1. Experimental procedure for *in vivo* trial



a) start of Cr-mordanted lucerne hay introduction
b) start of PEG infusion
c) start of $Na_2{}^{35}SO_4$ infusion
d) cessation of marker introduction
e) feces collection period
f) duodenal digesta sampling period
g) rumen sampling for bacteria, VFA and ammonia
h) rumen sampling for the determination of marker dilution rates

Table 4-1 : Protozoal population in the rumen of faunated sheep (x 10^4 / ml of rumen fluid)

species	(L)	(S)	SED	
Entodinium sp.	62.70	26.48	3.30	P<0.001
Polyplastoron multivesiculatum	2.95	1.72	0.17	P<0.001
Isotricha sp	0.69	0.66	0.15	P<0.05
Total protozoa	66.34	28.86	3.50	P<0.001

L,S: details are shown in Table 3-1

of 30g PEG per day per animal. These two markers were used simultaneously and the introduction of these markers began one week before the sampling period. $Na_2{}^{35}SO_4$ was included into the solution of PEG at 3 days before the collection of duodenal digesta and its infusion continued for 9 days at the rate of 250 μCi per day per animal. The first week (day 1 to day 7) of sampling period of each trial was used for feces collection. Duodenal digesta (70 ml) was taken every four hours on days 8 and 9 according to the sampling schedule shown in Figure 3-1. Half of each duodenal sample was filtered through 100 mesh(250 μm) immediately after being taken. Filtrates and whole digesta were then pooled separately and stored at -20°C. Rumen contents (400 ml) were taken every two hours for ten hours on the day 10. Sample of rumen bacteria were obtained by differential centrifugation as described in chapter 3 and freeze-dried immediately. Rumen contents (250 ml) were taken at 1,2,4,8,12,24,and 30 hours after the withdrawal of these three markers on the day 13 and 14 and stored at -20°C. During the faunated period, the protozoal number was determined by the method of JOUANY et al. (1981) on samples taken two hours after feeding for five successive days.

Chemical analysis

DM content was calculated after drying at 80°C for 48 hours and OM content was calculated after ashing at 600°C for six hours. Total nitrogen was determined by the kjeldhal method.

Ammonia nitrogen in rumen and duodenal contents was determined as described by WEATHERBURN (1967). Ruminal VFA analysis was carried out as described by JOUANY (1982). PEG was determined turbidimetrically as described by MALAWER and POWEL (1967). Cr^{3+}, PB, DAP and organic ^{35}S were measured as described in chapter 3.

Caluculations

The amount of each constituent entering the duodenum was estimated using the dual-marker technique (FAICHNEY,1975) based on 100 % recovery of both PEG and Cr. Estimation of microbial-N (MN) flow was made by dividing the amount of microbial marker entering into the duodenum by the ratio of the same marker to nitrogen content in bacteria. Rumen undegraded feed protein was estimated by substraction of MN and endogenous-N from duodenal non-ammonia nitrogen (NAN). Endogenous-N flow was estimated as 1 g per day (WESTON and HOGAN,1967). Rumen fractional turnover rate was estimated by fitting the marker concentration to the equation ; $C = Co\ e^{-kt}$ (FAICHNEY,1975).

Statistical analysis

The data from one sheep fed (S) in the first trial of the defaunated period was eliminated because of feed refusal. Therefore the values were analyzed according to the analysis of variance for unbalanced data (GILL 1978). The comparison

between the MN flow estimation by three markers method was achieved by one way analysis of variance.

Table 4-2 Volatile fatty acids (VFA) and ammonia concentration in defaunated and faunated sheep

Diet (1)	Animals (2)	NH_3-N (mg/ℓ) (min-max) (3)		Total VFA (mM/ℓ)	molar %					
					C_2	C_3	iC_4	C_4	iC_5	C_5
L	DF	54.1	(17.8-93.3)	74.5	64.97	22.26	0.62	9.67	0.90	1.23
L	F	139.9	(97.8-181.8)	82.4	66.37	17.71	0.70	12.83	0.69	1.32
S	DF	98.0	(78.8-126.8)	57.9	72.12	19.87	0.52	5.96	0.56	0.88
S	F	190.8	(125.0-237.0)	65.9	70.77	16.99	0.73	9.51	0.67	1.09
sem		12.9		3.8	0.71	0.65	0.03	0.71	0.06	0.05
Statistical significance (4)										
DF vs. F		**		NS	NS	**	**	**	NS	*
L vs. S		**		*	**	NS	NS	**	NS	**

(1) Details are shown in Table 3-1

(2) DF: defaunated, F: faunated.

(3) (min-max) : minimum and maximum observed values.

(4) * P< 0.05, ** P<0.01, NS : not significant

RESULTS

There was no protozoal contamination throughout the defaunated period. The (L) fed and (S) fed sheep had, respectively, 7.6×10^5 ml^{-1} and 3.5×10^5 ml^{-1} of protozoa during the faunated period. Dietary effect on the quantitative protozoal population size was highly significant ($p<0.01$). The Entodinium species accounted for more than 90 % of the species mixture of protozoa with both diets. Polyplastron multivesiculatum accounted for 5 to 6 % of population and holotrichs accounted for only 0.5 to 1 % of population.(Table 4-1). The (L) diet favoured the development of Entodinium sp. and P. multivesiculatum but holotrichs was not affected by the diet.

Rumen fermentation pattern is shown in Table 4-2. Ammonia level in the defaunated rumen was lower than that in the faunated rumen ($p<0.01$) and it was lower in the rumen of sheep fed (L) than that of sheep fed (S) ($p<0.01$). Extremely low rumen ammonia level was recorded in some defaunated sheep fed (L) ; 17.8, 28.0, 49.0 mg NH_3-N/ l. Ruminal total VFA concentration was lower in defaunated sheep than in faunated sheep, but the difference was not statistically significant. Dietary effect on total VFA was clear ; (L) promoted significantly higher total VFA concentration than did (S). In the molar proportion of VFA mixture, the defaunation increased the propionic acid and decreased the butyric acid and valeric acid. The diet had a clear effect on acetic acid butyric acid and valeric acid, but it had little effect on

Table 4-3. Dry matter(DM) and Acid detergent fiber(ADF) digestion in defaunated and faunated sheep

(1) Diet	(2) Animals	(3) DMi (g/ d)	DM apparently digested (g/ d) in rumen	in intestine	whole tract digestibility(%)	(3) ADFi (g/ d)	digested ADF(g/ d)	ADF digestibility(%)
L	DF	1107	253	386	57.7	323	123	38.1
L	F	1178	416	253	59.8	335	145	43.1
S	DF	1119	285	391	57.4	474	270	56.9
S	F	1184	384	309	58.5	459	270	58.9
SEM(4)		8	17	17	5.3	15	15	2.1
statistical significance(5)								
DF vs. F		NS	**	**	NS	NS	*	*
L vs. S		**	NS	NS	NS	**	**	**

(1) details are shown in Table 3-1

(2) DF: defaunated sheep, F: faunated sheep

(3) DMi: DM intake, ADFi: ADF intake

(4) standard error of mean

(5) * $p<0.05$,** $p<0.01$, NS: not significant

Table 4-4. Organic matter(OM) digestion in defaunated and faunated sheep

(1) Diet	(2) Animals	(3) OMi (g/ d)	OM apparently digested (g/ d) in rumen	OM apparently digested (g/ d) in intestine	whole tract digestibility(%)	OM truly digested in the(4) rumen (% OMi) PB	^{35}S	DAP	SEM(5)
L	DF	1019	299	302	59.0	44.8	48.3	47.6	1.0
L	F	1032	448	186	61.4	57.2	60.5	57.0	1.1
S	DF	1059	324	294	59.2	48.6	48.2	48.9	1.1
S	F	1064	415	220	59.7	55.1	55.1	54.5	1.4
SEM(5)		4	15	13	0.5	1.5	1.6	1.1	
statistical significance(6)									
DF vs. F		**	**	**	NS	**	**	**	
L vs. S		**	NS	NS	NS	NS	NS	NS	

(1) details are shown in Table 3-1
(2) DF: defaunated sheep, F: faunated sheep
(3) OMi: OM intake
(4) There was no statistical significant difference ($p>0.05$) between microbial markers
(5) standard error of mean
(6) * $p<0.05$, ** $p<0.01$, NS: not significant

Table 4-5. Nitrogen digestion(g N/d) in defaunated and faunated sheep

(1) Diet	(2) Animals	(9) Ni	duodenal N flow(3) TN	duodenal N flow(3) NAN	duodenal MN flow (4) PB	duodenal MN flow (4) ^{35}S	duodenal MN flow (4) DAP	duodenal MN flow (4) SEM	rumen undegraded feed N flow (4)(5) PB	rumen undegraded feed N flow (4)(5) ^{35}S	rumen undegraded feed N flow (4)(5) DAP	rumen undegraded feed N flow (4)(5) SEM	fecal N excretion	N digestibility(%) whole tract	N digestibility(%) (6) intestine
L	DF	22.1	33.6	32.8	18.1$^{\alpha}$	14.7$^{\alpha}$	17.7$^{\alpha}$	1.1	13.7$^{\alpha}$	17.1$^{\alpha}$	14.1$^{\alpha}$	0.8	8.6	61.1	73.8
L	F	22.8	24.2	23.4	15.3$^{\alpha}$	12.6$^{\alpha\beta}$	12.1$^{\beta}$	0.5	7.1$^{\alpha}$	9.8$^{\alpha}$	10.3$^{\alpha}$	0.7	8.0	64.9	65.8
S	DF	21.8	31.1	29.9	18.5$^{\alpha}$	18.8$^{\alpha}$	19.2$^{\alpha}$	1.2	10.5$^{\alpha}$	10.1$^{\alpha}$	9.8$^{\alpha}$	0.9	8.0	63.3	73.2
S	F	23.7	25.1	23.8	16.1$^{\alpha}$	16.2$^{\alpha}$	15.5$^{\alpha}$	0.9	6.7$^{\alpha}$	6.7$^{\alpha}$	7.3$^{\alpha}$	0.7	7.4	68.8	68.9
SEM(7)		0.2	1.1	1.1	1.0	0.8	0.8		0.9	1.1	0.8		0.1	0.7	1.0
statistical significance(8)															
DF vs. F		**	**	**	NS	NS	**		**	**	*		**	**	**
L vs. S		NS	NS	NS	NS	*	NS		NS	**	**		**	*	NS

(1) details are shown in Table 3-1, (2) DF: defaunated sheep, F: faunated sheep, (3) TN: total N, NAN: non ammoniacal N, (4) different superscripts indicate statistical difference($p<0.05$) between microbial markers, MN: microbial N, (5) endogenous N estimated as 1 g N/ d, (6) NAN digestibility in in the intestine, (7) standard error of mean, (8) * $p<0.05$, ** $p<0.01$, NS: not significant
(9) Ni: N intake

propionic acid and branched chain VFA. With the treated straw, the acetic acid proportion was higher whilst that of butyric acid and valeric acid were lower.

The DM and ADF digestibilities are shown in Table 4-3. The DM digestibilities in whole digestive tract were similar for two types of animal, although the ruminal DM digestion was depressed by defaunation. While ADF digestibility was depressed by defaunation. The OM digestibilities in the whole digestive tract were similar for the two diets(Table 4-4). Although the defaunation depressed ruminal OM digestion, whole tract digestibility was not affected by the defaunation. This indicates a shift in the digestion from the rumen to the intestines induced by the defaunation.

Nitrogen flowing into the duodenum was significantly higher in defaunated sheep than in faunated sheep($p<0.01$)(Table 4-5). A net gain in nitrogen between mouth and duodenum was observed in the defaunated sheep (approximately 10 g nitrogen per day). MN flowing into the duodenum was higher in defaunated sheep than those in faunated sheep. However the differences were not significant, except when the MN was estimated from DAP method. Therefore, the defaunation increased considerably the amount of feed nitrogen which escaped the rumen degradation. Concerning the dietary effect on MN flow, (S) promoted a slightly larger MN flow than did (L). The rumen non degraded feed nitrogen was higher with (L) diet than with (S) diet. Fecal nitrogen

Table 4-6. Efficiency of microbial protein synthesis and feed N degradability in defaunated and faunated sheep

(1) Diet	(2) Animals	efficiency of microbial protein synthesis(g Ni/kg OMDR)(3)(4)				feed N degradability in the rumen(4) (% N intake)			
		PB	^{35}S	DAP	SEM	PB	^{35}S	DAP	SEM
		α	α	α		α	α	α	
L	DF	63.0	50.5	60.6	5.0	38.1	22.5	36.0	3.8
		β	α	α		α	α	α	
L	F	34.2	28.1	26.9	1.2	69.0	57.1	54.8	2.8
		α	α	α		α	α	α	
S	DF	57.2	58.2	59.2	3.7	52.0	53.8	55.3	4.1
		α	α	α		α	α	α	
S	F	38.7	38.5	37.3	1.5	71.6	71.5	68.8	3.2
SEM(5)		4.4	3.2	3.8		4.1	4.9	3.6	
statistical significance(6)									
DF vs. F		**	**	**		**	**	**	
L vs. S		NS	NS	NS		NS	**	**	

(1) details are shown in Table 3-1, (2) DF: defaunated sheep, F: faunated sheep (3) OMDR: organic matter apparently digested in the rumen, Ni: N incorporated into microbial protein, (4) different superscripts indicate statistical difference ($p<0.05$) between microbial markers, (5) standard error of mean, (6) * $p<0.05$, ** $p<0.01$, NS: not significant

output was slightly but significantly higher in defaunated sheep ($p<0.01$), consequently nitrogen digestibility was decreased by defaunation independently of the diet. However, despite this point, the amount of NAN absorbed in the intestines is much higher in defaunated than in faunated sheep($p<0.01$). However, it was not known which part of intestine, small or large, had more important role on nitrogen disapperance in this study.

Efficiency of MN synthesis and feed degradability in the rumen are shown in Table 4-6. The comparison of the three different microbial markers did not show a statistical difference between the methods except the difference between PB and DAP methods in the faunated sheep fed (L). MN synthesis in defaunated sheep were 1.5 to twice more efficiently than those in faunated sheep. Treated straw promoted higher efficiency than (L) did in the faunated sheep, while the diet had little effect in defaunated sheep. Feed nitrogen degradability was affected by the defaunation. Degradability of feed nitrogen in faunated rumen was between 1.3 to 2 times as much as in defaunated rumen. According to the predicted solubility, nitrogen degradability of (S) was about 1.5 times higher than that of (L)(0.43 vs. 0.25).

Chemical composition of bacteria was affected by the defaunation(Table 4-7). The nitrogen, nucleic acid and DAP contents were higher in bacteria taken from defaunated sheep than those taken from faunated sheep. The dietary effect on bacterial chemical composition was less clearer than the protozoal effect.

Table 4-7. Chemical composition of bacteria(g/ kg DM)

(1) Diet	(2) Animals	OM	N	(3) PB	DAP
L	DF	890	84	91.0	3.5
L	F	873	79	72.5	2.8
S	DF	878	87	98.3	3.2
S	F	867	81	85.7	2.5
SEM(4)		4	1	3.6	0.1
statistical significance(5)					
DF vs. F		NS	*	**	**
L vs. S		NS	NS	*	NS

(1) details are shown in Table 3-1, (2)DF: defaunated sheep, F: faunated sheep, (3) purine base content is expressed as g of yeast RNA (RNA eq), (4) standard error of mean, (5) * $p<0.05$, ** $p<0.01$, NS: not significant

Turnover rate and retention times of rumen liquid phase (estimated by PEG dilution rate), of solid phase (estimated by chromium dilution rate), and of microbial proteins (estimated by organic ^{35}S dilution rate) are shown in Table 4-8 and in Figures 4-1a and 4-1b. All three fractions retained for a longer time in faunated rumen than in defaunated rumen. The dietary effect was also observed on the liquid and solid phase retention times. Organic ^{35}S concentration decreased at similar rate to Cr in defaunated sheep, but ^{35}S retained much longer than did Cr in faunated sheep (Figures 4-1a and 4-1b). There was no significant effect of protozoa or diet on the rumen fluid volumes and on rumen DM pool size.

DISCUSSION

(L) promoted a larger protozoal population than (S) due to its higher starch content. This difference was mainly due to the increase in the number of Entodinium sp., and biomass of this protozoa in the rumen of sheep fed high starch ration in limited amount is always important (e.g. JOUANY et al.,1977; JOUANY and SENAUD,1982). On the other hand, holotrichs number was not affected by dietary conditions. Entodinium sp. are considered as most sensible species to the dietary change (JOUANY,1978; DENNIS et al.,1983), while holotrichs number does not greatly vary according to the dietary change (EADIE et al.,1970; NAKAMURA and KANEGASAKI,1969; DEHORITY and MATTOS,1978; DENNIS et al.,1983).

The present results confirm these observations.

Effect of defaunation on end products of rumen fermentation

The effect of defaunation on ruminal ammoniogenesis and VFA production, decreasing both ammonia and total VFA concentration, agree with previously published results (see COLEMAN,1981; DEMEYER,1981;JOUANY et al.,1981). Ingestion of bacteria and proteolytic action on dietary protein by protozoa promote greater rumen ammonia concentration in faunated animals. Protozoa cannot utilize ingested protein efficiently ; only 50 % of ingested bacterial nitrogen is retained in the protozoon and the remainders return to the surrounding environment as the form of amino acid which are further catabolized to ammonia by the bacteria (COLEMAN,1975). (L) promoted lower ammonia concentration, especially in defaunated rumen. Since (L) contained less soluble nitrogen and greater readily fermentable carbohydrate than (S), ammoniogenesis from dietary nitrogen degradation may be smaller and bacterial ammonia assimilation rate may be greater with (L) than (S).

The negative effect of defaunation on total VFA concentration in this study is related to the reduced ruminal OM digestion (Table 4-3,4). The reduction of butyric acid concentration agree with most of observations done in defaunated rumen (Table 1-1). Since protozoa produce acetic acid and butyric acid (HUNGATE,1966), increased butyric acid concentration in faunated

rumen is partly due to the protozoal butyric acid production. The increase observed in the propionic acid molar proportion in defaunated animals is probably induced by a change in rumen flora which can be seen through the evolution of bacterial chemical composition (Table 4-7). Bacteriological studies showed that acetic acid producing species, such as Ruminococcus, are not predominant species, while succinate producers such as Bacteroides are predominant in defaunated calves (BRYANT and SMALL, 1960). Amylolytic species increase in defaunated rumen; mainly B.ruminocola and B.amylophilus which produce succinate (KURIHARA et al.,1978). These replacement may enhance propionic acid production. DAP content in bacterial preparations taken from the faunated rumen was significantly lower than that in bacteria taken from the defaunated rumen. Among the most abundant amylolytic species; Streptococcus bovis, B. amylophilus, B. ruminocola, and Selenomonas ruminantium, only S.bovis has the gram positive cell wall structure. It is, therefore, suspected that reduction of DAP content in bacterial preparations indicates the reduction of amylolytic species.

Protein degradation and role of microbial flora

Both MN flow and dietary nitrogen which escaped from ruminal degradation increased in the defaunated sheep. If the endogenous nitrogen flow was not greatly affected by the defaunation, the extent of increase in rumen undegraded dietary

nitrogen was much greater than that in MN flow (4 g N d^{-1} vs 2 g N d^{-1}). These *in vivo* results showing that the dietary nitrogen degradability is significantly decreased agree with the observations obtained by an *in vitro* and a nylon bag technique in chapter 2. Protozoa can ingest directly small particulate matter, such as bacteria, starch granules (COLEMAN and HALL,1969) and chloroplast (MANGAN and PRYOR,1968; WEST and MANGAN,1972) and their proteolytic activity has been widely demonstrated (WARNER,1956; WILLIAMS et al.,1961; ABOU AKKADA and HOWARD,1962; NAGA and EL SHAZLY,1968). Therefore higher dietary nitrogen degradability in faunated rumen is, at least in part, due to the protozoal proteolysis. The change of bacterial flora composition, as discussed earlier, induced by the defaunation would affect the proteolytic activity in the rumen. However the relationship between the proteolytic activity and the type of flora composition was always unclear. SIDDONS and PARADINE (1981) suggested that proteolytic activity depends on the number of bacteria rather than on the type of flora. The results shown in chapter 2 confirm this suggestion.

GANEV et al.(1979) suggested that the accesibility of microbial protease to the dietary protein, especially insoluble proteins, is a main factor to determine the rate of degradation of dietary protein. This accesibility is closely related to the OM or cellulose digestibility. In the present study, OM digestion in the rumen of faunated sheep was more intensive than in the

rumen of defaunated sheep. In another study, JOUANY and SENAUD (1979b) reported a higher level of cellulose digestion in the faunated rumen compared to that in the defaunated rumen. Therefore insoluble protein might be more accesible to the microbial protease in the faunated sheep rumen. Retention time of feed protein in the rumen is another factor which determines the ruminal insoluble protein degradability. When turnover rate of rumen contents is high, low protein degradability is observed (TAMMINGA et al.,1979; ØRSKOV et al.,1980). In our study, the mean retention time of mordanted chromium is longer in faunated than in defaunated rumens, but the difference between two types of animals is small: 3.5 hours for the sheep fed (L) and 1.8 hours for the sheep fed (S). The lucerne hay mordanted with chromium was ground through a 4 mm screen before being incorporated in the pelleted diet. With such a treatment, the lucerne became friable and it is likely that very small particles were obtained during grinding. With these conditions, chromium probably labelled small and very small particles in the rumen, the later being largely composed of microorganisms. This assumption is confirmed by the similar retention times for ^{35}S and mordanted chromium in defaunated animals. With roughage diets such as those used in this study, liquid and solid phases are distinct whereas they are intimately mixed in concentrate diets. Therefore, the mean retention time of whole solid phase is plobably longer than that of the small particles measured here with

Table 4-8. Turnover rate and retention time of liquid, solid fraction and microbial markers in defaunated and faunated sheep rumen

(1) Diet	(2) Animals	Turnover rate (h^{-1}) PEG	mordanted Cr^{3+}	organic ^{35}S	Retention time (h) PEG	mordanted Cr^{3+}	organic ^{35}S	rumen fluid volume(ℓ)	rumen DM pool(kg)
L	DF	0.0807	0.0654	0.0726	12.5	15.6	13.9	5.8	0.49
L	F	0.0641	0.0546	0.0377	15.9	19.1	27.6	5.9	0.57
S	DF	0.0963	0.0794	0.0677	10.4	12.7	15.0	5.6	0.42
S	F	0.0881	0.0706	0.0448	11.4	14.5	22.1	6.8	0.51
SEM(3)		0.0031	0.0029	0.0035	0.5	0.8	1.4	0.3	0.03
statistical significance (4)									
DF vs. F		**	*	**	**	**	**	NS	NS
L vs. S		**	**	NS	**	**	NS	NS	NS

(1) details are shown in Table 3-1, (2) DF: defaunated sheep, F: faunated sheep.

(3) standard error of mean, (4) *p<0.05,**p<0.01, NS: not significant

the mordanted chromium.

Influence of protozoa on mean retention time

After inoculation with protozoa, the mean retention time of PEG and mordanted chromium is increased. These results obtained with roughage based diets suggest that protozoa increase the retention of liquid and small particles in the rumen. This finding agrees with the results reported by KAYOULI et al.(1983,1984) obtained on a straw diet, but is contrary to the observations of FAICHNEY and GRIFFITHS (1978) who observed the opposite effect with a concentrate diet. It is still difficult to explain the effect of protozoa on the retention time of either fluid or solid phase of rumen contents. The protozoa can be sequestered in attaching them to the rumen wall or to large feed particles (ABE et al.,1981; BAUCHOP and CLARKE,1976; ORPIN 1979a and b). They ingest, and therefore sequester, small feed particles, so that sequestration may delay the retention time of small feed particles. Contrary to present results, ORPIN and LETCHER (1984) reported recently an increase in retention time of liquid phase of rumen contents after the defaunation. However their results were obtained from the measurement of marker disapperance rate in the rumen of sheep fed once daily and 6 hours before the feeding. Therefore it is difficult to compare their results with present results obtained from sheep under quite different feeding management.

Figure 4-1a. Marker disappearance rate in the rumen of defaunated sheep

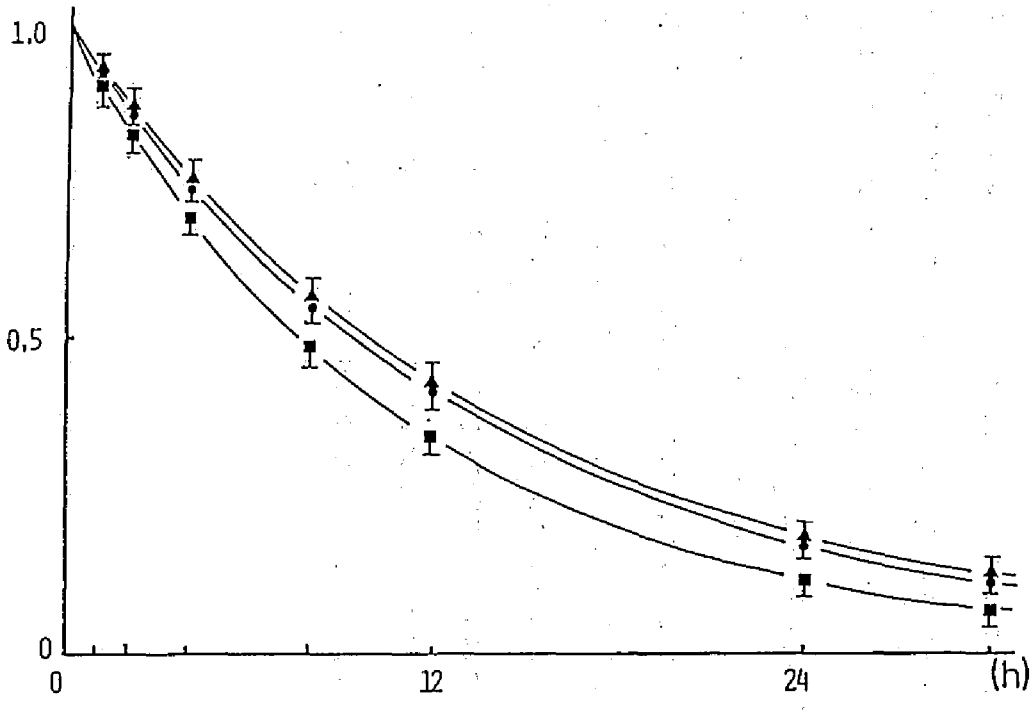
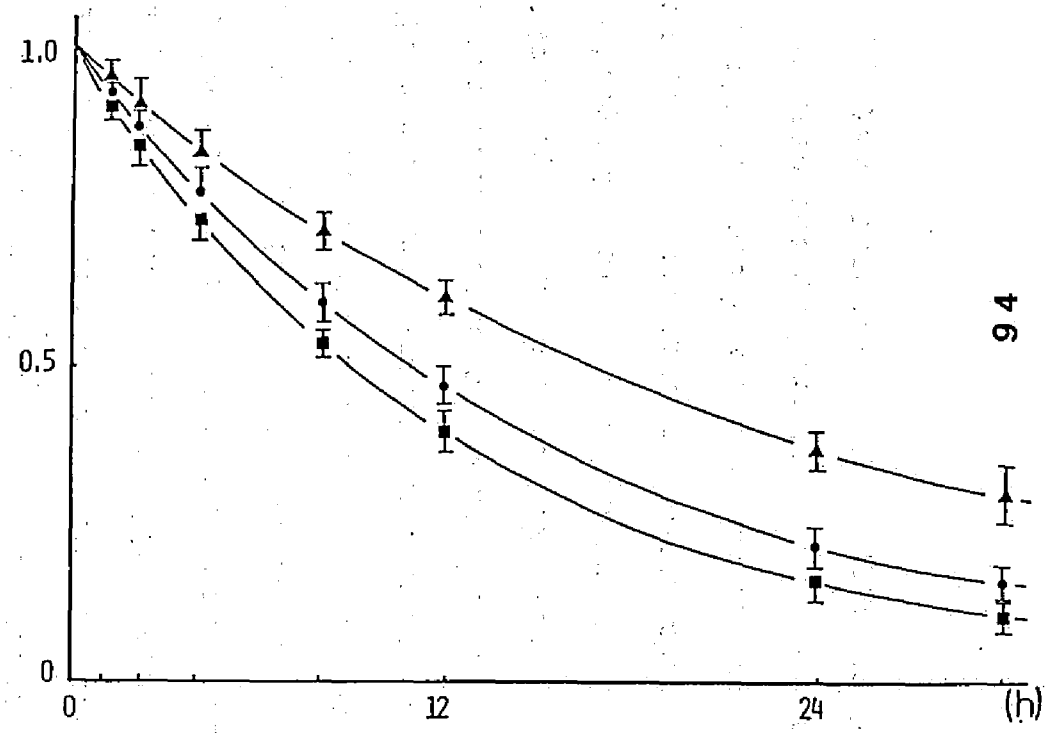


Figure 4-1b. Marker disappearance rate in the rumen of faunated sheep



X: Time (hour) after cessation of marker distribution,

Y: Marker concentration relative to 0 time concentration

Figure 4-1a

The mean curves of marker disappearance from rumen were established using the pooled results taken from six defaunated sheep fed (L) and five defaunated sheep fed (S). Turnover rates for all markers are shown in Table 4-8 Organic ^{35}S (▲), mordanted Cr^{3+} (●), PEG (■). Vertical bars denote standard error.

Figure 4-1b

The mean curves of marker disappearance from rumen were established using the pooled results taken from six faunated sheep fed (L) and six faunated sheep fed (S). Turnover rates for all markers are shown in Table 4-8 Organic ^{35}S (▲), mordanted Cr^{3+} (●), PEG (■). Vertical bars denote standard error.

Generally speaking, the microorganisms flow out from the rumen in two ways. They are associated with liquid phase or attached to the feed particles. Therefore microbial marker would give a middle value of turnover rate between those of the liquid phase marker and solid phase marker. However measured ^{35}S turnover rate was similar to mordanted chromium turnover rate in defaunated sheep and slower than that of mordanted chromium in faunated sheep. The recycling of ^{35}S within the rumen, lowered its turnover rate. The magnitude of this recycling is not known but it may be emphasized in faunated rumen because of the protozoal predation of bacteria. Although the proportion of two types of bacteria, associated with liquid or solid phase, which flow out from the rumen is not exactly known, the results obtained in defaunated sheep could indicate that larger part of bacteria which flow out from the rumen consisted of small particles associated bacteria because the recycling of bacterial matter in the rumen of these animals is fairly small (DEMEYER and VAN NEVEL,1979). This could also indicate that the outflowed rumen digesta mainly contains liquid associated bacteria if we considered that the mean retention time of large particles is longer than that measured by mordanted chromium. Comparing the retention time of ^{35}S in defaunated and faunated animals, we can conclude that protozoa increase the retention time of microorganisms in the rumen which can be explained by the sequestration of protozoa(WELLER and PILGRIM, 1974 ; HARRISON and McALLAN,

1980 ; COLLOMBIER,1981).

Influence of protozoa on microbial protein synthesis

Bacterial nitrogen flow which was estimated by DAP was higher in defaunated sheep comparing to that in faunated sheep. Bacterial protein synthesis efficiency was also highest in defaunated sheep. The efficiency measured in faunated sheep is close to the standard value (INRA,1978; ARC, 1980). The same results were obtained in MN flow and MN synthesis efficiency when they were estimated both by PB and ^{35}S method. These results indicate that protozoal nitrogen supply could not compensate for the decrease of bacterial nitrogen flow in faunated sheep. DEMEYER and VAN NEVEL (1979) showed that the efficiency of bacterial protein synthesis was increased by defaunation in an *in vitro* experiment. They suggested that the lower bacterial degradation in the defaunated rumen, induced by the disappearance of predation, increases the efficiency of microbial synthesis. As discussed earlier, the disappearance of predation and nutritional competition by defaunation promote a larger bacterial population which directly explains the larger bacterial cell outflow.

Moreover, a higher turnover rate of liquid phase of rumen contents was observed in defaunated animals. The increase of rumen fluid turnover rate, which was induced experimentally by the introduction of buffer salts or by the cold exposure of animal, improve the efficiency of microbial protein synthesis in

the rumen (HARRISON et al.,1975,1976; KENNEDY and MILLIGAN, 1978). Therefore the modification observed in the turnover rate of liquid or solid phase in the rumen can explain the evolution of the bacterial protein synthesis efficiency.

Comparison of the three methods used for the estimation of microbial synthesis and of protozoal contribution to duodenal nitrogen

The three different markers used in this study to measure the microbial nitrogen showed relatively good agreement and particularly when comparison were carried out with defaunated animals, the only case where it is possible to do it. No statistical difference were detected, except between PB and DAP method in the faunated sheep fed (L). However the variations between the animals were sometimes relatively high. The reliability of microbial markers has been strongly discussed in the recent decade (e.g. HARRISON and McALLAN, 1980). Since it is impossible to verify the results obtained by any marker methods using an absolute reference, only a comparative study allows to access the verification of marker reliability. Many results taken from the simultaneous marker comparison studies have been accumulated (LING and BUTTERY,1978; SMITH et al.,1978; SIDDONS et al.,1979,1982; MERCER et al.,1980), but it is still difficult to draw a definitive conclusion because of some discrepancies between the results. For the nucleic acid method, the dietary conta-

mination in duodenal nucleic acids has been pointed out. MN flow estimated by RNA method showed usually highest value. A PB determination was proposed recently by ZINN and OWENS (1980) who verified the reliability of this method using a protein free semipurified diet. It was observed that this method had given a similar value to those estimated by DAP and ^{35}S in defaunated sheep even with more conventional diet(chapter 3). The present results also show, on the whole, a relatively good reliability of this method. However there was some discrepancies between the ^{35}S method and the other two methods in defaunated sheep fed (L), but it is impossible to conclude if ^{35}S method did underestimate microbial synthesis in this study. In the present study, the protozoal nitrogen contribution to MN in duodenum was 20 % with PB-DAP comparison in the sheep fed (L), while 4 % of MN was attributable to the protozoa in the sheep fed (S). This value observed in sheep fed (L) is similar to previously published results taken from sheep with same size of rumen protozoal population (HARRISON et al.,1979 ; SUTTON et al.,1983), but further investigation is needed to verify these estimations by specfic marker of protozoa such as phosphatidyl choline (JOHN and ULYATT,1984).

SUMMARY

The role of protozoa on rumen digestion, in vivo, was studied using 6 defaunated and 6 faunated sheep. The sheep were fed two isonitrogenous diets containing either lucerne hay (Diet L) or NaOH treated wheat straw (Diet S), which had different nitrogen solubilities (25.0 with L, 43.0 with S). Diet L promoted a large protozoal population, while S supported a smaller protozoal population. Sheep were fed eight equal portions at 3 hour intervals, mean daily intake of DM was 53 g / kg $BW^{0.75}$. Digesta flow from forestomach was estimated by spot sampling of duodenal digesta using dual marker technique with Cr^{3+} mordanted lucerne hay and PEG. The microbial protein flow at duodenum was estimated using PB, ^{35}S, and DAP, simultaneously. The different microbial markers were compared in the defaunated sheep and protozoal contribution was estimated in the faunated sheep.

Defaunated sheep had lower ruminal ammonia and butyrate concentrations, but they had higher propionate concentration than faunated sheep. These observations agree with those in chapter 1. Rumen DM and OM digestion were depressed by defaunation, but these depression were compensated for by increased intestinal digestion. ADF digestion, however, was not compensated by intestinal digestion. A net increase of nitrogen flow occurred between mouth and duodenum in defaunated sheep independently of diet, indicating a large quantity of recycled nitrogen was fixed in the rumen. Both microbial (bacterial) and dietary protein entering

the duodenum were larger in defaunated sheep than in faunated. Microbial protein synthesis efficiencies (g N i/ Kg OMDR) in defaunated sheep were twice as high as those in faunated sheep, latter values are similar to those published by INRA and ARC as a mean value. Dietary protein degradabilities (% N intake) were lower in defaunated sheep compared to those in faunated sheep. However, relative values (DF/F) in protein degradability were higher with diet S than with diet L, these results indicate that protozoa have a larger effect on insoluble protein degradation than on soluble one. These observations agree with those in chapter 2. Protozoa prolong the mean retention times of solid and liquid phase, explaining, at least in part, increased dietary protein degradation in the rumen.

GENERAL DISCUSSION AND CONCLUSION

In this thesis, a series of experiments were carried out in order to study the contribution of protozoa to ruminant digestion, especially to protein digestion. Role of protozoa on rumen feed protein degradation, on bacterial protein synthesis, and on rumen carbohydrate fermentation were investigated in this study.

Being based on the observations in this study, role of rumen protozoa on ruminant digestion is classified into following five heads.

1. Effect of protozoa on dry matter (DM) and organic matter (OM) digestion of the ruminant

Ruminal DM and OM digestion were depressed by defaunation, but the increase in the DM and OM digestion in the intestine compensated these decreases (Table 4-3,4-4). Whole tract DM and OM digestibilities, thus, were not affected by defaunation. Contrary to DM or OM, ADF digestibility in whole tract was depressed significantly by defaunation. Cellulose digestion in the rumen would be depressed by defaunation and intestinal ADF digestion could not compensate such a decrease. This suggests that protozoa have a considerable effect on cellulose digestion. Since some Ophryoscolecidae, Polyplastron multivesiculatum and Entodinium sp., were demonstrated to have cellulase, these protozoa contribute directly to rumen cellulose digestion. It has been previously reported that protozoa stimulate bacterial activity by the predation. Protozoal contribution to cellulose digestion is

considered as indirect in this case. Thus, protozoa enhance ruminal DM, OM and cellulose digestion in two way, direct and indirect.

2. Effect of protozoa on the end products of rumen fermentation

Defaunation decreased the production of acetate, butyrate, carbon dioxide and methane, while it increased propionate production in the rumen(Table 1-3,4,4-2). These changes, in the amount of end products, observed in defaunated sheep were derived from the changes of bacterial flora composition and from the disappearance of protozoal acetate, butyrate and hydrogen production, latter will be further utilized in methane formation. These results and depressed cellulose digestion in defaunated sheep suggest that protozoa encourage acetate producing cellulolytic species, such as Ruminococcus sp. and limit the proliferation of propionate, lactate or succinate producing amylolytic species such as Selenomonas and Bacteroides. Change in bacterial flora composition is also suggested by changes in chemical composition of bacterial preparations(Table 4-7). It is suggested that protozoa play an important role on the establishment of acetogenic fermentation in the rumen of animal which is fed a high concentrate diet and has a large protozoal population.

Hydrogen produced by protozoa is, at least in part, supplied to methanogen, because electromicroscopic studies demonstrated the colony of methangen on protozoal surface. Thus proto-

zoa enhance methane formation. Total VFA production was decreased slightly by defaunation. This decrease is mainly based on depressed DM digestion in the rumen (Table 1-3,4-2).

Ammonia concentration in defaunated sheep rumen was considerably low (Table 4-2). Only 50 % of protein of ingested bacteria is retained in protozoa and the remainders return to the surrounding environment as the form of amino acid which are further catabolized to ammonia. Increase in the lysis of bacteria and in the feed protein degradation under the presence of protozoa promote higher ammonia concentration compared to that in defaunated sheep rumen.

3. Effect of protozoa on protein digestion in the rumen

Protozoa enhance considerably degradation of dietary protein in the rumen(Table 4-5,6) and their role is more important on the insoluble protein degradation than on soluble one (Table 2-1,2,3,4). Soluble protein attach onto the microbial surface and bacterial protease and peptidase which locate on the bacterial surface degrade them. Protozoa have a different mode of protein degradation : Protozoa ingest small feed particles and digested in their cytopharynx where the relative enzyme concentration in respect to substrate is higher than surrounding environment. Since protozoa stimulate OM and cellulose digestion, nitrogenous compounds contained in structural components of feed are more accesible to the microbial enzyme.

4. Effect of protozoa on bacterial protein synthesis

Bacterial nitrogen flowing into duodenum was two fold larger in defaunated sheep than in faunated sheep(Table 4-5). Efficiencies of bacterial protein synthesis(g Ni / kg OMDR) in defaunated sheep rumen were also two fold more efficient compared to those in faunated sheep rumen which were similar to those previously presented as mean value(30g Ni/kg OMDR). These results suggest that protozoa diminish bacteria which flow out from rumen by decreasing rumen bacterial concentration. Moreover protozoal contribution to duodenal microbial protein was small, 4-20 % of duodenal microbial nitrogen (Table 4-5), protozoa cannot compensate the reduction of bacterial nitrogen flow. Thus, increased bacterial nitrogen flow and decreased OM digestion in the rumen explain such a high efficiency. A net increase in non ammonia nitrogen(NAN) between mouth and duodenum was observed in defaunated sheep(Table 4-5). If the amount of endogenous nitrogen flow is not affected by defaunation, these net increase indicate an efficient endogenous non-protein-nitrogen (NPN) fixation by bacteria in defaunated sheep rumen.

5. Influence of protozoa on mean retention time

Protozoa increased mean retention time of PEG, Cr^{3+} mordanted lucerne hay pellet, and ^{35}S. These results suggest that protozoa increase retention time of both liquid and particle phase of rumen contents. Protozoa ingest small feed particles and

then sequester, the sequestration may delay the retention time of particle phase. Increased ^{35}S retention time is explained by the sequestration of protozoa which are marked by ^{35}S and by amplified recycling of ^{35}S in the rumen under the presence of protozoa. Increased retention time of feed particle promotes a net increase in ruminal degradation of feed.

Finally protozoal contribution to the ruminant digestion is concluded as follows.

Protozoa decrease the amount of both bacterial protein and rumen non degraded feed protein which reach to the duodenum. For the rumen feed protein degradation, protozoa play an important role on insoluble protein degradation, but they little contribute to the soluble protein degradation. For the rumen bacterial protein synthesis, protozoa decrease the efficiency(g Ni/kg OMDR) and decrease the rate of fixation of endogenous NPN by bacteria. It is, therefore, certain that protozoa have a negative effect on the ruminant protein nutrition.

Protozoa enhance acetate, butyrate ,carbon dioxide and methane production in the rumen. Defaunation, then, increased propionate production and decreased methane production. Therefore, defaunation can improve the energetic efficiency of feed. However protozoa enhance cellulose digestion in the rumen and defaunation usually decreases total VFA production. If the animals fed a diet containing large amount of ligno-cellulose, defaunation can provoke an important loss of energy, because

total VFA production would be fairly depressed by defaunation under such a dietary condition. It is acceptable to consider that defaunation improves energetic efficiency of feed when the animals are fed a diet containing considerable amount of readily fermentable carbohydrate which assures that total VFA production is not too depressed by defaunation. In conclusion, defaunation may have a positive effect on the animal growth and production when animals are fed a diet containing high readily fermentable carbohydrate and limited amount of protein. Particularly for certain products which depend more on the intestinal protein supply, such as wool production, may be easily improved their productive efficiency by defaunation. However defaunation must have a negative effect on milk fat synthesis which depends on rumen butyrate production.

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* * *

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REFERENCES

ABE M.,T.IRIKI,N.TOBE and H.SHIBUI (1981) Appl.Environ.Microbiol. 41,758-765.

ABOU AKKADA A.R. and B.H. HOWARD (1962) Biochem.J.,82,313-320.

ABOU AKKADA A.R. and K. EL-SHAZLY (1964) Appl.Envioron.Microbiol 12,384-390.

ABOU AKKADA A.R. and K.EL-SHAZLY (1976) In Tracer Studies on Non Protein Nitrogen for Ruminants.III. pp 49-56. IAEA. Vienna.

ANNISON E.F. (1956) Biochem. J.,64,705-714.

A.R.C. (1980) The Nutrient Requirements of Ruminant Livestock. C.A.B., Slough U.K.

BAUCHOP T. and R.T.J. CLARKE (1976) Appl.Environ.Microbiol.,32, 417-422.

BEEVER D.E.,D.G.HARRISON,D.J.THOMSON,S.B.CAMMEL and D.F.OSBOURN (1974) Br.J.Nutr.,32,99-112.

BIRD S.H. and R.A. LENG (1978) Br.J.Nutr.,40,163-167.

BIRD S.H., H.K.HILL and R.A.LENG (1979) Br.J.Nutr.,42,81-87.

BIRD S.H. and R.A.LENG (1984) Br.J.Nutr.,52,607-611.

BERGEN W.G.,D.B.PURSER and J.H.CLINE (1968) J.Anim.Sci.,27,1497-1501.

BORHAMI B.E.A.,K.EL-SHAZLY,A.R.ABOU AKKADA and I.A.AHMED(1967) J.Dairy Sci.,50,1654-1660.

BROCK F M.,C.W.FORSBERG and J.G.BUCHANAN SMITH (1982) Appl. Environ.Microbiol.,44,561-569.

BRYANT M.P. and N. SMALL (1960) J.Dairy Sci.,43,654-667.

CHALMERS M.I.,J.DAVIDSON, J.M.EADIE and J.C.GILL (1968) Proc.Nutr Soc.,27,29A-30A.

CHRISTIANSEN W.C., R. KAWASHIMA and W. BURROUGHS (1965) J.Anim. Sci.,24,730-734.

COCKBURN J.E. and A.P.WILLIAMS (1984) Br.J.Nutr.,51,111-132.

COLEMAN G.S. (1975) In Digestion and Metabolism in the Ruminant. pp 149-164.(McDONALD I.W. and WARNER A.C.I., eds.) Univ. New England Publishing Unit.Armidale,NSW,Australia.

COLEMAN G.S. (1979) Trop.Anim.Prod.,4,199-213.

COLEMAN G.S. (1981) Adv. Parasitol.,18,121-163.

COLEMAN G.S. and F.J. HALL (1969) Tissue and Cell 1,607-618.

COLLOMBIER J.(1981) Thèse de docteur ingenieure.Univ.CLERMONT II, N°d'ordre 105.

CZERKAWSKI J.W.,(1976) J.Sci.Food Agric.,27,621-632.

DEHORITY B.A. and W. MATTOS (1978) Appl.Environ.Microbiol.,36, 953-958.

DEMEYER D.I. (1981) Agriculture Environment 6,295-337.

DEMEYER D.I. and C.J. VAN NEVEL (1975) In Digestion and Metabolism in the Ruminant pp366-382.(McDONALD I.W.and A.C.I.WARNER eds.) Univ.New England Publishing Unit. Armidale,NSW,Australia.

DEMEYER D.I. and C.J. VAN NEVEL (1979) Br.J.Nutr.,42,515-524.

DEMEYER D.I., C.J.VAN NEVEL and VAN DE VOORDE (1982) Arch. Tierernahrung 32,595-604.

DENNIS S.M., M.J.ARAMBEL, BARTLEY E.E. and DAYON A.D. (1983) J.Dairy Sci.,66,1248-1258.

EADIE J.M. and P.N.HOBSON (1962) Nature,193,503-505.

EADIE J.M.,J.HYLDGAARD-JENSEN,MANN S.O.,REID R.S. and F.G.WHITE-LAW (1970) Br.J.Nutr.,24,157-177.

EADIE J.M. and J.L. GILL (1971) Br.J.Nutr.,26,155-167.

ELLIS W.C. and W.H.PFANDER (1965) Nature, 205,974-975.

ERFLE J.D.,R.J.BOILA,R.M.TEATHER,S.MAHADEVAN and F.D.SAVER(1982) J.Dairy Sci.,62,1457-1464.

FAICHNEY G.J. (1975) In Digestion and Metabolism in the Ruminant. pp261-276.(McDONALD I.W. and A.C.I. WARNER eds.) Univ.New England Publishing Unit. Armidale, NSW, Australia.

FAICHNEY G.J. and D.A. GRIFFITHS (1978) Br.J.Nutr.,40,71-82.

GALE E.F. and J.P.FOLKES (1953) Biochem J.,53,483-493.

GANEV G.,E.R.ØRSKOV and R.SMART (1979) J.Agric.Sci.Camb.,93,651-656.

GAUSSÈRES B.and G.FAUCCONEAU (1966) Ann.Biol.anim.Biochem.Biophys 5,5-20.

GILL J.L. (1978) Design and Analysis of Experiments in the Animal and Medical Science vol.I pp 239-248. Iowa State Univ. Press. Ames, Iowa,USA.

GRUBY D. and O.DELAFOND (1843) cited by HUNGATE (1966).

HA J.K. and J.J. KEENELLY (1984) Can.J.Anim.Sci.,64,791-793.

HARRISON D.G.,D.E.BEEVER,D.J.THOMSON and D.F.OSBOURN (1975) J.Agric.Sci.Camb.,85,93-101.

HARRISON D.G.,D.E.BEEVER,D.J.THOMSON and D.F.OSBOURN (1976) J. Sci.Food Agric.,27,617-620.

HARRISON D.G., D.E.BEEVER and D.F.OSBOURN (1979) Br.J.Nutr.,41, 521-527.

HARRISON D.G. and A.B.McALLAN (1980) In Digestive Physiology and Metabolism in Ruminant pp205-226(RUCKEBUSH Y., P.THIVEND eds.) MTP Press. Lancaster. U.K.

HIBBS J.W. and H.R.CONRAD (1958) J.Dairy Sci.,41,1230-1248.

HUNGATE R.E. (1966) Rumen and Its Microbes. Academic Press,N.Y.

HUNGATE R.E., J.REICHL and R.PRINS (1971) Appl.Microbiol.,22,1104 -1113.

IBRAHIM E.A. and J.R. INGALLS (1972) J.Dairy Sci.,55,971-978.

I.N.R.A.(1978) Alimentationdes Ruminants, INRA public. Versailles, France.

ITABASHI H.,T.KOBAYASHI and M.MATSUMOTO (1984) Jpn.J.Zootech.Sci. 55,248-256.

JEENER R. (1953) Arch.Biochem.Biophys.,43,381-388.

JOHN A. and M.J. ULYATT (1984) J.Agric.Sci.Camb.,102,33-44.

JOUANY J.P.(1978) Thèse de Docteur es Sciences, Univ.CLERMONT II, N d'ordre 256.

JOUANY J.P. (1982) Sciences des Aliments 2,131-141.

JOUANY J.P. and P.THIVEND (1972) Ann.Biol.anim.Biochem.Biophys., 12,673-677.

JOUANY J.P., J.SENAUD, J.GRAIN, P.DE PUYTRAC and P.THIVEND (1977) Proc.Nutr.Soc.,36,72A.

JOUANY J.P. and J.SENAUD (1979 a) Ann.Biol.anim.Biochem.Biophys. 19,619-624.

JOUANY J.P. and J.SENAUD (1979 b) Ann.Rech.Vét.,10,261-263.

JOUANY J.P., B. ZAINAB, J.SENAUD, C.A.GROLIÈRE, J.GRAIN and P.THIVEND (1981) Reprod.Nutr.Develop.,21,874-884.

JOUANY J.P. and J.SENAUD (1982) Reprod.Nutr.Dévelop.,23,607-623.

JOUANY J.P. and J.SENAUD (1983) Reprod.Nutr.Dévelop.,23,607-623.

KAYOULI C.,C.VAN NEVEL and D.I.DEMEYER (1983) In Metabolism et Nutrition azotées. vol.II,(Les colloques de l'INRA) INRA ed. pp 251-254.

KAYOULI C.,D.I.DEMEYER,C.J.VAN NEVEL and R.DENDOOVEN (1984) Anim.Feed Sci.Technol.,10,165-172.

KENNEDY P.M. and L.P. MILLIGAN (1978) Br.J.Nutr.,39,105-117.

KURIHARA Y.,J.M.EADIE,P.N.HOBSON and S.O.MANN (1968) J.Gen. Microbiol.,51,267-288.

KURIHARA Y.,T.TAKECHI and F.SHIBATA (1978) J.Agric.Sci.Camb.,90, 373-382.

LENG R.A. (1982) Br.J.Nutr.,48,399-415.

LENG R.A.,M.GILL,T.J.KEMPTON,I.B.ROWE,J.V.NOLAN,S.J.SACHIW and T.R.PRESTON (1981) Br.J.Nutr.,46,371-384.

LINDSAY J.R. and J.P. HOGAN (1972) Aust.J.Agric.Res.,23,321-330.

LING J.R. and P.J.BUTTERY (1978) Br.J.Nutr.,39,165-175.

LORING H.S. (1955) In The Nucleic Acids vol.I (CHARGAFF E. and J.N.DAVIDSON eds.) pp 191-199. Academic Press N.Y.

LUTHER R.,A.TRENKLE and W.BURROUGHS (1966) J.Anim.Sci.,25,1116-

1122.

McALLAN A.B.and SMITH R.H.(1969) Br.J.Nutr.,23,671-682.

McALLAN A.B. and R.H.SMITH (1972) Proc.Nutr.Soc.,31,24A.

McALLAN A.B. and SMITH R.H.(1973) Br.J.Nutr.,29,467-474.

McALLAN A.B. and SMITH R.H.(1974) Br.J.Nutr.,31,77-88.

McALLAN A.B. and SMITH R.H.(1983) Br.J.Nutr.,49,119-127.

McALLAN A.B. and R.H.SMITH (1984) Br.J.Nutr.,51,77-83.

McDONALD I.W. (1954) Biochem.J.,57,566-568.

McNAUGHT M.L.,E.C.OWEN,K.M.HENRY and S.K.KON (1954) Biochem.J., 56,151-156.

MALAWER S.J. and D.W. POWEL (1967) Gastroenterology 53,250-256.

MANGAN J.L. and M.J. PRYOR (1969) J.Physiol.,200,18p-19p.

MASON V.C. and S.BECH-ANDERSEN (1976). Z.Tierphysiol.Tierernah-rg.u.Futtermittelkd.,36:221-229.

MATHERS J.C. and E.L.MILLER (1980) Br.J.Nutr.,43,503-514.

MATHIESON J. (1970) Proc.Nutr.Soc.,29,30A.

MERCER J.R.,ALLEN S.A. and MILLER E.L. (1980) Br.J.Nutr.,43,421-433

MICHALOWSKI T. (1979) J.Agric.Sci.Camb.,93,67-70.

MITCHELL P. and J. MOYDE (1951) J.Gen.Microbiol.,5,421-435.

NAGA M.A. and K. EL-SHAZLY (1968) J.Gen.Microbiol.,53,305-315.

NAKAMURA K. and S.KANEGASAKI (1969) J.Dairy Sci.,52,250-255.

NUGEANT J.M. and J.L. MANGAN (1981) Br.J.Nutr.,46,39-58.

OGIMOTO K. and S.IMAI (1981) Atlas of Rumen Microbiology. Japan Scientific Press, Tokyo.

ONODERA R. and M. KANDATSU (1970) Jpn.J.Zootech.Sci.,41,307-313.

ORPIN G.C. (1979 a) Soc.Gen.Microbiol.Quart.,7,31-32.

ORPIN G.C. (1979 b) Soc.Gen.Microbiol.Quart.,7,32.

ORPIN G.C. and A.J. LETCHER (1984) Anim.Feed Sci.Technol.,10,145-153.

ØRSKOV E.R. and I.McDONALD (1979) J.Agric.Sci.Camb.,92,499-503.

ØRSKOV E.R.,M.HUGHS-JONES and I.McDONALD (1980) In Recent Advances in Animal Nutrition (1980) pp85-98 (HARESIGN W.,ed.) Butterworth London U.K.

POUNDEN W.D. and J.W. HIBBS (1950) J.Dairy Sci.,33,639-644.

PURSER D.B. and S.M. BUECHLER (1966) J.Dairy Sci.,49,81-84.

SCWEINSBERG P.D. and TI LI LOO (1980) J.Chromatog.,181,103-107.

SIDDONS R.C.,D.E.BEEVER,J.V.NOLAN,A.B.McALLAN and J.C.Mac RAE (1979) Ann.Rech.Vét.,10,286-287.

SIDDONS R.C. and J.PARADINE (1981) J.Sci.Fd.Agric.,32,973-981.

SIDDONS R.C.,D.E.BEEVER and J.V.NOLAN (1982) Br.J.Nutr.,48,377-389.

SMITH R.H.(1969) J.Dairy Res.,36,313-331.

SMITH R.H., A.B.McALLAN, D.HEWITT and P.E.LEWIS (1978) J.Agric. Sci.Camb.,90,557-568.

SNEDECOR G.W. and W.G.COCHRAN (1967) Statistical Methods,6th edition, The IOWA State Univ.Press,Ames,Iowa,USA.

STUMM C.K.,H.J.GIJZEN and G.D.VOGELS (1982) Br.J.Nutr.,47,95-99.

SUTTON J.D.,R.KNIGHT,A.B.McALLAN and R.H.SMITH (1983) Br.J.Nutr., 49,419-432.

TAMMINGA S.,C.J.VAN DER KOELEN and A.M.VAN VUUREN (1979) Livestock Prod.Sci.,6,255-262.

THIVEND P. and J.P.JOUANY (1985) submitted to Anim.Feed Sci.Tech.

TOPPS J.M. and ELLIOT R.C.(1965) Nature, 205,498-499.

UDEN P.,P.E.COLUCCI and P.J.VAN SOEST (1980) J.Sci.Food Agric., 31,625-632.

VALDEZ R.E., F.J.ALVAREZ,H.M.FERREIRO,F.GUERRA,J.LOPEZ,A.PRIEGO, T.H.BLACKBURN,R.A.LENG and T.R.PRESTON (1977) Trop.Anim.Prod., 2, 260-272.

VEIRA D.M.,M.IVAN and P.Y.JUI (1984) Can.J.Anim.Sci.,64,22-23.

VERITÉ R. and C. DEMARQUILLY (1978) In La Vache Laitière.pp143-157. INRA public. Versailles,France.

WARNER A.C.I.(1956) J.Gen.Microbiol.,14,749-762.

WEATHERBURN M.W. (1967) Analytic. Chem.,39,971-974.

WELLER R.A. (1957) Aust.J.Biol.Sci.,10,384-389.

WELLER R.A., F.V.GRAY and A.F.PILGRIM (1958) Br.J.Nutr.,12,421-429.

WELLER R.A. and A.F.PILGRIM (1974) Br.J.Nutr.,32,341-351.

WEST J. and J.L. MANGAN (1972) Proc.Nutr.Soc.,31,108A-109A.

WESTON R.H. and J.P. HOGAN (1967) Aust.J.Biol.Sci.,20,967-973.

WHITELAW F.G.,J.M.EADIE, S.O.MANN and R.S.REID (1972) Br.J.Nutr., 27,425-437.

WHITELAW F.G.,J.M.EADIE,L.A.BRUCE and W.J.SHAND (1984) Br.J.Nutr. 52,261-275.

WILLIAMS P.D. R.E. DAVIS, R.N.DOETSH and J.GUTIERREZ (1961) Appl.Microbiol.,9,405-409.

WILLIAMS P.P. and W.E. DINNUSON (1973) J.Anim.Sci.,36,588-591.

WOLIN M.J.(1974) Am.J.Clin.Nutr.,27,1320-1328.

WORK E. and D.DEWEY (1953) J.Gen.Microbiol.,9,394-409.

ZINN R.A. and F.N.OWENS (1980) In Protein Requirement for Cattle. pp26-30.1980 Protein Symposium, OKLAHOMA State Univ.Stillwater Oklahoma.USA.

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